Control de la podredumbre por Botrytis cinerea mediante la aplicación de Candida sake CPA-1 y otras estrategias alternativas a los fungicidas químicos en uva de vinificación

Carlos Calvo Garrido

Dipòsit Legal: L.410-2014
http://hdl.handle.net/10803/134607

Universitat de Lleida
Departament de Tecnologia
d'Aliments

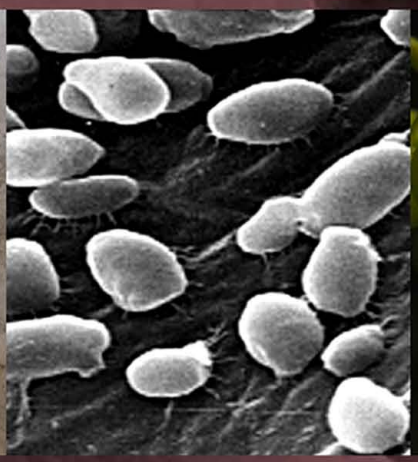
Control de la podredumbre por *Botrytis cinerea* mediante la aplicación de *Candida sake* CPA-1 y otras estrategias alternativas a los fungicidas químicos en uva de vinificación

Tesis Doctoral

Carlos Calvo Garrido

Lleida, 2013






Universitat de Lleida

Escola Tècnica Superior d'Enginyeria Agrària

Departament de Tecnologia d'Aliments

Control de la podredumbre por *Botrytis cinerea* mediante la aplicación de *Candida sake* CPA-1 y otras estrategias alternativas a los fungicidas químicos en uva de vinificación

Memoria presentada por:

Carlos Calvo Garrido

Para optar al grado de:

Doctorado en Ciencia y Tecnología Agraria y Alimentaria

Directoras de Tesis:

Neus Teixidó i Espasa

Inmaculada Viñas Almenar

Lleida, Noviembre de 2013

A mis padres, por darme todo su apoyo y motivarme para hacer esta tesis. Pero sobre todo por estar siempre de mi lado y comprender mi forma de ver las cosas, que a veces no es poco.

A los familiares y los buenos compañeros que ya no están, porque sé que si me vieran estarían muy contentos por mí.

Control de la podredumbre por *Botrytis cinerea* mediante la aplicación de *Candida sake* CPA-1 y otras estrategias alternativas a los fungicidas químicos en uva de vinificación

AGRADECIMIENTOS

Después de cuatro años de experiencias y trabajo, unas páginas no van a ser capaces de recoger la importancia que ha tenido toda la gente con la que he compartido este tiempo. Sin ell@s habría sido completamente imposible llegar a ningún puerto. Todo el trabajo científico, su comprensión y por supuesto el poder disfrutar de ello, depende en gran medida de haber tenido a mi alrededor un montón de personas increíbles que, cada una a su manera, me han permitido progresar hasta terminar este documento. Muchas gracias a todos, moltes gràcies a tothom, many thanks to everyone, merci beaucoup à tous!

A Neus Teixidó, por la dedicación y el tiempo empleado con este trabajo en todo momento, aunque fuera sacándolo de debajo de las piedras. No todo el mundo tiene la suerte de tener una directora de tesis que se asegure de que todo salga bien y eso se nota. Por su energía y profesionalidad, porque trabajar con una persona tan competente le empuja a uno a hacer las cosas mejor. Pero sobre todo por apostar por mí para realizar la tesis, haber tenido paciencia conmigo cuando las cosas salían bien del todo y darme su confianza para realizar dos estancias y hacerme responsable de los trabajos de investigación. Por supuesto también por preocuparse de mi salud y mis ánimos cuando han flojeado. Por esto y muchas cosas más… ¡muchas gracias Neus!

A Inmaculada Viñas, por haber estado siempre pendiente de mis necesidades y del progreso de la tesis, participando también de los contenidos de la investigación a pesar de las mil responsabilidades y asuntos de los que se ocupa cada día. Por su solvencia para arreglar cualquier problema en un par de horas, por hacer que todo haya ido sobre ruedas en mi relación con la UdL y por preocuparse de sus doctorandos y avisarnos de cada convocatoria u oportunidad para ayudarnos.

A Josep Usall, por haber estado siempre dispuesto a ayudar cuando más lo he necesitado, por implicarse en el proceso de investigación y los resultados, por su visión a largo plazo del trabajo y por implicarse también en mis proyectos de futuro en la investigación. Y en lo personal, por preocuparse de que todo me iba bien en todo momento, por ayudarme a reenfocar los problemas cuando han surgido y por tener siempre una palabra de ánimo.

To Phil Elmer, because he has been a huge referent and a guide in my research on *Botrytis* and alternative control methods, helping me to learn a lot and transmitting his passion, dedication and creative thinking in science. But, above all, because he has really become a very good friend in which I can count on, although we are few miles away from each other. Also to the Elmer family (Julie, Zoe, Aleysha and little Charlie), because they really made me feel at home during my stay in New Zealand, taking care of me as if I was a close relative and enjoying their time with me. I really appreciate your hospitality and friendship. I hope we will see each other soon!

To the Biological Control and Natural Products group at the Ruakura research centre, Hamilton, New Zealand: Frank, Kirstin, Annette, Tony, Joe, Mike, for their help in my research and in my every-day life in NZ, and for those lovely coffee times. I'm especially thankful to Dr. Steve Hoyte for his thorough revision of the thesis manuscript, for his hospitality during my

stay and for our nice coffees and conversations in the office about science and Rock&Roll. Also to Rob Agnew, Lynne, Peter Wood and his family, for their warm welcome in Marlborough and Hawks bay during field sampling journeys.

À Marc Fermaud pour son invitation à travailler avec lui dans le centre de recherche INRA Bordeaux-Aquitaine, qui a était un vrai plaisir et une expérience inoubliable. Pour son intérêt dans mon investigation, pour sa calme et sa vision bien médité de la recherche et pour les intéressantes conversations sur la science, la politique et la vie. Aussi pour son invitation à réaliser un projet postdoctoral avec lui, qui reste toujours une grande fierté pour moi.

À tous les collègues de l'UMR-SAVE, surtout a Jean, Lionel, Jérôme, Sylvain, Olivier, Sylvie, Patrice, Juliette, Pascale, Jessica, Pierre, Amira et Rezk, pour son accueil hyper gentil, son aide avec les manips et pour les bons moments qui on a passé pendant la saison d'été de 2012. Bien sûre aussi aux copains faites à Bordeaux qui m'ont fait trop «kiffer» mon stage en France et avoir une récompense après les longes journées de travail : la maison Pichounette & Co. (Maria & Vincent, Julie, Philipe « Phacó » et José) et la « communauté internationale » de Bordeaux, surtout à Giovanni parce que il n'y a pas un autre comme lui, même si il est « fait en bois », et à Jose y la Cánovas por su arte y por abrirme las puertas de su casa y de de ellos mismos, que eso no puede pagarse con nada.

A Charo Torres y Maribel Abadías, por estar siempre dispuestas a echar una mano con lo que sea y por sus orientación y ayuda de primer nivel en cuestiones científicas, como corresponde a dos pedazo de investigadoras como son ellas.

A Cristina, por ser un amor de persona con un gran sentido del humor y por estar siempre pendiente de que todo salga bien en la investigación de todo el mundo, desde luego en la mía un montón. Me has dado más cobertura que una agencia de seguros y que un ángel de la guarda: con la logística del laboratorio, ayudando en los ensayos, discutiendo los resultados, supervisando la planificación (controlando mi caos)… También por preocuparse de que te sientas cómodo en el plano personal y por aguantar la embestida del toro cuando he perdido los nervios. Ets molt gran Cris! (molt gran però sobre tot molt jove).

A mis hermanitas doctorandas (aunque algunas ya ostenten el título de Doctoras!) con las que he compartido alegrías, penas, tabaco, risas, lágrimas, delirios, cervezas, cenas, agobios, enchiladas y bailes: Carla, Gemma, Isa, Laura, Lulú, Marcia, María Bernat, María Sisquella, Pilar y Vivi. Por supuestísimo al gran Jaime Cano "Ho Chi Mihn" por las horas y horas de risas y arreglar el mundo. Muchas gracias a tod@s por vuestro cariño y por preocuparos tanto de mi y muchas fuerza y ánimos para terminar la tesis a las que todavía estáis en ello, ha sido un placerazo estar estos años con vosotr@s.

A Robert, Cèlia, Neus Lamarca, Marina, Rosa Altissent, Rosa Vilaplana, Lucía, Sveta, a todo el equipo del STP, al departamento de fisiología y a Gemma Echeverria, porque con vosotr@s he compartido también el día a día y de cada un@ he aprendido alguna cosa. También por los buenos ratos que hemos pasado hablando de cualquier tema y porque en más de una ocasión me habéis salvado el pellejo en el laboratorio o fuera de él.

A Mariela por su ayuda durante toda la dura campaña de 2011 y por ser tan cariñosa y tan buena que hemos llegado a tener una conexión increíble a pesar de ser muy diferentes en muchas cosas. Todo lo mejor para ti y tu familia y ¡felicidades por tu tesis!

Al equipo de la cooperativa "L'Olivera", sobre todo a Pau, Carles, Clara e Isabelle, por su implicación en el proyecto y su ayuda logística en los ensayos de campo durante toda la tesis. Gracias también por mantener un proyecto como el vuestro, que consigue hacer un mundo más respetuoso con la tierra y con las personas… y por hacer un producto de primera, ¡claro!

A Xavi Ochoa, Santi y Elena Cases por el trabajo realizado durante las campañas 2009 y 2010, que forma el grueso de los resultados de esta tesis y que no habría sido posible sin su esfuerzo.

A mi familia, especialmente a mis padres y a mi abuela, porque han vivido con muchísima ilusión estos años aunque la tesis me alejara de ellos, porque comprendieron rápidamente que era lo que yo quería hacer, que es bueno para mí y han disfrutado de mis historias y de verme contento en Lleida, Nueva Zelanda y Francia. Por estar siempre ahí queriéndote y respondiendo por lo que sea, porque eso te hace feliz y seguro de ti mismo y cuando uno se siente así, el resto de cosas salen rodadas en la vida.

A toda la gente con la que he tenido el placer de compartir techo en estos 4 años: Guillem, Ainhoa Zabaleta, Ainhoa Ibarrola, Miren, Bea, Raúl, Juanjo, Hans, Ilaria, Maria, Matu, Laura y todo el resto de la CasaBlava Crew, porque han hecho que mi vida en Lleida haya sido una experiencia increíble, me han hecho feliz en momentos en que no era fácil y han sido los mejores compañeros de viaje para aprender muchísimo sobre la vida y sobre uno mismo… puff cuánto os debo, algún día dentro de unos años quizá termine de asimilar todo lo que habéis supuesto para mí. También al resto de amigos con mayúsculas que me quedan en Catalunya para siempre y que he tenido el placer de conocer en estos años en las Terres de Ponent, sobre todo a Miguelo, Kim y Ari (ànims amb la tesi megacrack!).

A la gran familia de Madrid, porque a pesar de la distancia han seguido siendo un gran respaldo y me han hecho sentirme querido: al Gordo, Jimi, Manu y Fraguas (una promesa es una promesa) y todos mis eskautbroders, a NATAS por sacar siempre lo mejor de mí, a los Malayerberos por seguir igual de cracks después de unos cuantos años, en especial a Kike por su amistad incondicional y a Irene por estar conmigo a full en momentos muy duros de la tesis y por un millón de cosas más, a los velilleros por recordarme quien soy (especialmente al Piña por sus mil visitas) y a los compis del Ghetto del Pilar. De todos ellos, además, a Alba, Ainhoa, Nuri, el Moro, Virginia y Chema, porque ha sido un lujo y un gran apoyo tener cerca de Lleida a hermanitos "de toda la vida".

A la UdL, por el proyecto de investigación, la beca predoctoral y la beca para la estancia en Nueva Zelanda, sin las cuales no habría sido posible la realización de este trabajo.

A todas las personas que no he nombrado pero que también son parte de esta tesis, porque con cada persona que compartes algo aprendes alguna cosa y eso forma parte del proceso.

Control de la podredumbre por *Botrytis cinerea* mediante la aplicación de *Candida sake* CPA-1 y otras estrategias alternativas a los fungicidas químicos en uva de vinificación

Los trabajos presentados en esta tesis se han desarrollado en el laboratorio de Patología de la Post-cosecha del centro IRTA (Institut de Recerca i Tecnologia Agroalimentàries) de Lleida, en el marco científico de los dos siguientes proyectos de investigación:

"Proyecto de cooperación transfronteriza de agricultura ecológica" REDBIO INTERREG (Programa de Cooperación España-Francia-Andorra). Financiado por la Unión Europea.

"Control de las principales podredumbres de melocotón, nectarina y uva de vinificación en fruta de agricultura ecológica". Financiado por la Universidad de Lleida, dentro de la convocatoria de proyectos de investigación en producción y alimentación ecológica. Este proyecto de la UdL ha financiado asimismo la beca pre-doctoral de Carlos Calvo Garrido.

Control de la podredumbre por *Botrytis cinerea* mediante la aplicación de *Candida sake* CPA-1 y otras estrategias alternativas a los fungicidas químicos en uva de vinificación

ABREVIATURAS / ABREVIATURES / ABBREVIATIONS

ACB	Agente de Control Biológico
a_ω	Actividad de agua / Activitat d'aigua / Water activity
BCA	Biological Control Agent
BBR	*Botrytis* bunch rot
CFU	Colony Forming Units
FC	Fungicover®
h	Horas / Hores / Hours
HR	Humedad Relativa / Humitat Relativa
IRTA	Institut de Recerca I Tecnologia Alimentàries
NP	Natural Product
PBC	Podredumbre por *Botrytis cinerea*
PN	Producto Natural / Productes Naturals
PRT	Protector^{HML}
RH	Relative Humidity
T	Temperatura / Temperature
UFC	Unidades Formadoras de Colonias / Unitats Formadores de Colònies

Control de la podredumbre por *Botrytis cinerea* mediante la aplicación de *Candida sake* CPA-1 y otras estrategias alternativas a los fungicidas químicos en uva de vinificación

ÍNDICE

Control de la podredumbre por *Botrytis cinerea* mediante la aplicación de *Candida sake* CPA-1 y otras estrategias alternativas a los fungicidas químicos en uva de vinificación

ÍNDICE

RESUMEN / RESUM / SUMMARY

Control de la podredumbre por *Botrytis cinerea* mediante la aplicación de *Candida sake* CPA-1 y otras estrategias alternativas a los fungicidas químicos en uva de vinificación

La podredumbre gris del racimo o podredumbre por *Botrytis cinerea* (PBC), es una enfermedad fúngica responsable de importantes pérdidas económicas en regiones vitícolas de zonas de clima templado, debido a pérdidas tanto en cantidad como en la calidad del vino elaborado. El uso de fungicidas químicos de síntesis está cada vez más restringido debido al continuo desarrollo de resistencia a estas sustancias por el patógeno y los métodos de control de la PBC en agricultura ecológica son muy reducidos. Por ello, en viticultura convencional y ecológica se necesitan actualmente alternativas para el control de la PBC en campo y algunas de las más estudiadas y que presentan mayor potencial son el control biológico con microorganismos y la aplicación de productos naturales.

El agente de control biológico (ACB) *Candida sake* CPA-1 es una levadura que ha demostrado reducir la PBC en cosecha cuando fue aplicada en campo junto con el aditivo Fungicover® (FC) en estudios previos. Sin embargo, varios aspectos referentes a la optimización de los tratamientos y su modo de acción, así como a la supervivencia de *C. sake* y los factores que la afectan están por determinar.

La presente tesis doctoral investiga estos aspectos con los siguientes objetivos: **1)** estudiar la eficacia de los tratamientos de *C. sake* con FC, comparando con otros productos comerciales para el control de la PBC y evaluando su eficacia en momento de cosecha y su control de fuentes de inóculo secundario del racimo (Cap. 1, 2, 4 y 6); **2)** Evaluar el efecto de estos tratamientos biológicos sobre otra enfermedad del racimo, la podredumbre ácida (Cap. 3); **3)** Investigar la epidemiología de la PBC en viñedos de climatología mediterránea (Lleida), determinando los momentos más importantes durante la campaña para el desarrollo de las fuentes de inóculo secundario y de la PBC en momento de cosecha (Cap. 2, 5); **4)** Evaluar el efecto de los factores que reducen la supervivencia de *C. sake* CPA-1 en campo, como Temperatura (T), Humedad Relativa (HR), lluvia o radiación solar (Cap. 6, 7).

Los experimentos de campo se realizaron durante dos campañas vitícolas (2009 y 2010) en Lleida (Catalunya, España) y una campaña en Bordeaux (Francia). Los tratamientos consistentes en 4-6 aplicaciones de *C. sake* ($5x10^7$ CFU g^{-1}) con FC (50 g L^{-1}) durante la campaña redujeron un 63-67 % de la incidencia y un 82-90 % de la severidad de la enfermedad, comparado con el control no tratado. Además, sólo dos aplicaciones, focalizadas en floración y pre cierre de racimo, consiguieron reducciones de la severidad similares a cuatro aplicaciones.

La combinación de *C. sake* y FC consiguió, de forma general, mejor eficacia que FC solo en ensayos de campo y laboratorio. Todos los tratamientos biológicos testados (*C. sake* CPA-1, FC, quitosán y *Ulocladium oudemansii*), así como las estrategias basadas en su combinación, redujeron significativamente la PBC en cosecha en todos los experimentos. Además, la severidad de la podredumbre ácida también fue reducida por los tratamientos incluyendo *U. oudemansii* (2009; 38-46 % de reducción comparado con el control) y *C. sake* (2009 y 2010; 40-66 % de reducción), lo cual representa el primer estudio que describe el control biológico de esta enfermedad.

En las campañas de 2009 y 2010, la incidencia de *B. cinerea* en tejidos necróticos del interior del racimo (caliptras, flores abortadas y frutos abortados) fue evaluada en momento de envero, comparando muestras no tratadas con muestras que habían recibido aplicaciones de productos biológicos. Los tratamientos con FC, *U. oudemansii* y quitosán redujeron la incidencia de *B. cinerea* en estos tejidos un 43-67 % comparado con el control, excepto en los frutos abortados.

Debido a las reducciones mostradas por los tratamientos con el producto basado en ácidos grasos FC, la eficacia y el modo de acción de este producto fueron estudiados específicamente, comparando con el producto comercializado Protector^{HML} (PRT). Cuando se aplicó en las dosis más altas, FC redujo la severidad de la infección por *B. cinerea* entre el 44 % y el 96 %, mientras que PRT presento en general una efectividad similar. Los resultados sugieren un modo de acción múltiple para FC, en el que la película establecida por FC sobre la superficie de bayas y hojas interfiere con la germinación de *B. cinerea*, bloquea infecciones por micelio y protege heridas. El modo de acción de PRT es similar, aunque no alteró la morfología del tubo germinativo y su protección de heridas fue limitada.

Durante las campañas de 2009 y 2010, se llevaron a cabo ensayos con diferentes calendarios de aplicación del fungicida iprodiona, aplicando en diferentes estados fenológicos de la viña. Los tratamientos incluyendo aplicaciones en floración fueron los más efectivos, sugiriendo que dos aplicaciones en floración son suficientes para controlar la PBC en las condiciones testadas, con la adición de otra aplicación post-envero si las condiciones meteorológicas son favorables para la enfermedad a partir del pre cierre de racimo. Además, se utilizaron las muestras del ensayo con fungicidas en 2010 para determinar la incidencia de diferentes fuentes de inóculo secundario de *B. cinerea* (infecciones latentes, tejidos necróticos del racimo e inóculo superficial). Los resultados mostraron que la mayoría de las infecciones latentes y de la colonización saprofítica de tejidos necróticos se produjo en floración. De este modo, tanto la evaluación de fuentes de inóculo como de la PBC en cosecha apuntaron la floración como el estado fenológico más importante para el desarrollo de la PBC. El análisis cuantitativo de estos datos evidenció correlación positiva entre tres fuentes de inóculo secundario (infecciones latentes, incidencia en caliptras y en frutos abortados) y la incidencia de PBC en cosecha. Estas tres variables se incluyeron en un modelo de regresión lineal múltiple, representando la primera publicación que relaciona varias fuentes de inóculo simultáneamente con la PBC en cosecha.

El estudio de los diferentes tejidos necróticos en bloques no tratados (dos campos experimentales ecológicos, 2009 y 2010) mostró que los frutos abortados presentaron una incidencia de *B. cinerea* significativamente más alta que las flores abortadas y las caliptras (38 %, comparado con 19 % y 24 %, respectivamente). Además, la incidencia en frutos abortados no fue reducida por los tratamientos biológicos, indicando que este tejido representa una importante fuente de inóculo. Sin embargo la abundancia de frutos abortados es generalmente reducida y dependiente del cultivar, por lo que la relevancia de este tejido para la PBC es variable.

Las poblaciones de *C. sake* sobre la superficie de las bayas fue monitorizada durante los ensayos de campo, recuperando las poblaciones de los bloques tratados después y justo antes de cada aplicación. Las poblaciones se mantuvieron entre 5.5 y 7 Log CFU g^{-1} sobre flores y 4-6 Log CFU g^{-1} sobre bayas. En las condiciones de clima mediterráneo de la zona de Lleida (2009 y 2010), las poblaciones decrecieron 1-2 log entre aplicaciones, mientras que no decrecieron en las condiciones de clima atlántico de los viñedos de Bordeaux. Estos resultados, junto con los obtenidos en los ensayos con regímenes climáticos simulados, confirmaron la importancia de las condiciones climáticas para la supervivencia de *C. sake* en campo.

La supervivencia de *C. sake* también fue evaluada en diferentes ensayos de campo y laboratorio, exponiendo las poblaciones a diferentes factores abióticos que habitualmente limitan la supervivencia de los ACBs, como T, HR, radiación solar y lluvia. Las poblaciones sobre bayas se redujeron 1-3 log en las primeras 24 horas de exposición a condiciones limitantes de alta T y baja HR, manteniendo poblaciones sobre el nivel de detección tras 72 horas de exposición. Por tanto, *C. sake* demostró su capacidad de supervivencia indicando el alto potencial de este ACB para su aplicación en campo. Las poblaciones de *C. sake* fueron muy sensible a los factores abióticos en el primer periodo tras la aplicación (6-24 horas), mientras que un periodo de establecimiento de las poblaciones en condiciones óptimas (24-48 horas; 20-22 °C y 85-100 % HR), previo a la exposición a lluvia simulada o condiciones limitantes, incrementó significativamente la persistencia y supervivencia de *C. sake*. Estos datos permitieron formular recomendaciones de aplicación en campo para este ACB.

En conclusión, los resultados presentados en esta tesis doctoral demuestran la alta eficacia de los tratamientos con *C. sake* CPA-1 y FC, que fue similar o mayor que la de otros productos registrados y en uso, confirmando la fiabilidad y el potencial de estos métodos de control biológicos para el control de la PBC en viña. Además, se han obtenido nuevos datos sobre la supervivencia de *C. sake* y la epidemiología de *B. cinerea*, valiosos para futuros estudios de control biológico de esta enfermedad. Esta información también revela importantes aplicaciones prácticas para optimizar los tratamientos con *C. sake* y otros medios de control alternativos a los fungicidas de síntesis.

Control de la podridura per *Botrytis cinerea* mitjançant l'aplicació de *Candida sake* CPA-1 i altres estratègies alternatives als fungicides de síntesi en raïm de vinificació

La podridura grisa del raïm o podridura per *Botrytis cinerea* (PBC), és una malaltia fúngica responsable d'importants pèrdues econòmiques en regions vitícoles de zones de clima temperat, a causa de pèrdues tant en quantitat com en la qualitat del vi elaborat. L'ús de fungicides químics de síntesi està cada vegada més restringit a causa del continu desenvolupament de resistència a aquestes substàncies per part del patogen i, a més a més, els mètodes de control de la PBC en agricultura ecològica són molt limitats. Per això, en viticultura convencional i ecològica es necessiten actualment alternatives per al control de la PBC a camp i algunes de les més estudiades i que presenten major potencial són el control biològic amb microorganismes i l'aplicació de productes naturals.

L'agent de control biològic (ACB) *Candida sake* CPA-1 és un llevat que ha demostrat reduir la PBC en collita quan va ser aplicada a camp amb l'additiu Fungicover® (FC) en estudis previs. No obstant això, diversos aspectes referents a l'optimització dels tractaments i el seu mecanisme d'acció, així com la supervivència de *C. sake* i els factors que l'afecten estan per determinar.

La present tesi doctoral investiga aquests aspectes amb els següents objectius: **1)** estudiar l'eficàcia dels tractaments de *C. sake* amb FC, comparant amb altres productes comercials per al control de la PBC i avaluant la seva eficàcia a collita i el seu control de fonts d'inòcul secundari del raïm (cap. 1, 2, 4 i 6), **2)** Avaluar l'efecte d'aquests tractaments biològics sobre una altra malaltia del raïm, la podridura àcida (Cap. 3); **3)** Investigar l'epidemiologia de la PBC en vinyes de climatologia mediterrània (Lleida), determinant els moments més importants durant la campanya per al desenvolupament de les fonts d'inòcul secundari i de la PBC en moment de collita (Cap. 2, 5); **4)** Avaluar l'efecte dels factors que redueixen la supervivència de *C. sake* CPA-1 a camp, com la Temperatura (T), la Humitat Relativa (HR), la pluja o la radiació solar (Cap. 6, 7).

Els experiments de camp es van realitzar durant dues campanyes vitícoles (2009 i 2010) a Lleida (Catalunya, Espanya) i una campanya a Bordeus (França). Els tractaments consistents en 4-6 aplicacions de *C. sake* ($5x10^7$ CFU g^{-1}) amb FC (50 g L^{-1}) durant la campanya van reduir un 63-67 % de la incidència i un 82-90 % de la severitat de la malaltia, comparat amb el control no tractat. A més a més, només dues aplicacions, focalitzades en floració i pre tancament de carroll, van aconseguir reduccions de la severitat similars a quatre aplicacions.

La combinació de *C. sake* i FC va aconseguir, de forma general, millor eficàcia que FC tot sol en assaigs de camp i laboratori. Tots els tractaments biològics testats (*C. sake* CPA-1, FC, quitosán i *Ulocladium oudemansii*), així com les estratègies basades en la seva combinació, van reduir significativament la PBC a collita en tots els experiments. D'altra banda, la severitat de la podridura àcida també va ser reduïda pels tractaments incloent *U. oudemansii* (2009; 38-46 % de reducció comparat amb el control) i *C. sake* (2009 i 2010; 40-66 % de reducció), la qual cosa representa el primer estudi en control biològic d'aquesta malaltia descrit fins el moment.

En les campanyes de 2009 i 2010, la incidència de *B. cinerea* en teixits necròtics de l'interior del raïm (caliptres, flors avortades i fruits avortats) va ser avaluada al moment de verolat, comparant mostres no tractades amb mostres que havien rebut aplicacions de productes biològics. Els tractaments amb FC, *U. oudemansii* i quitosan van reduir la incidència de *B. cinerea* en aquests teixits un 43-67 % comparat amb el control, excepte en els fruits avortats.

A causa de les reduccions mostrades pels tractaments amb el producte basat en àcids grassos FC, l'eficàcia i el mecanisme d'acció d'aquest producte van ésser estudiats específicament, comparant amb el producte comercialitzat Protector^{HML} (PRT). Quan es va aplicar a les dosis més altes, FC va reduir la severitat de la infecció per *B. cinerea* entre el 44 % i el 96 %, mentre que PRT va presentar en general una efectivitat similar. Els resultats suggereixen un mecanisme d'acció múltiple per a FC, en què la pel·lícula establerta per FC sobre la superfície del raïm i les fulles interfereix amb la germinació de *B. cinerea*, bloqueja infeccions per miceli i protegeix les ferides. El mecanisme d'acció de PRT és similar, encara que no va alterar la morfologia del tub germinatiu i la seva protecció de ferides va ser limitada.

Durant les campanyes de 2009 i 2010, es van dur a terme assajos amb diferents calendaris d'aplicació del fungicida iprodiona, tractant en diferents estats fenològics de la vinya. Els tractaments que incloïen aplicacions en floració van ser els més efectius, suggerint que dues aplicacions en floració són suficients per controlar la PBC en les condicions testades, amb l'addició d'una altra aplicació post-verolat si les condicions meteorològiques són favorables per a la malaltia a partir del pre-tancament de carroll. A més a més, es van utilitzar les mostres de l'assaig amb fungicides de 2010 per determinar la incidència de diferents fonts d'inòcul secundari de *B. cinerea* (infeccions latents, teixits necròtics del carroll i inòcul superficial). Els resultats van mostrar que la majoria de les infeccions latents i de la colonització saprofítica de teixits necròtics es va produir a floració. D'aquesta manera, tant l'avaluació de fonts d'inòcul com de la PBC en collita van assenyalar la floració com l'estat fenològic més important per al desenvolupament de la PBC. L'anàlisi quantitativa d'aquestes dades va evidenciar l'existència de correlació positiva entre les tres fonts d'inòcul secundari (infeccions latents, incidència en caliptres i en fruits avortats) i la incidència de PBC en collita. Aquestes tres variables es van incloure en un model de regressió lineal múltiple, que representa la primera publicació que relaciona diverses fonts d'inòcul simultàniament amb la PBC en collita.

L'estudi dels diferents teixits necròtics en blocs no tractats (dos camps experimentals ecològics, 2009 i 2010) va mostrar que els fruits avortats presentaven una incidència de *B. cinerea* significativament més alta que les flors avortades i les caliptres (38 %, comparat amb 19 % i 24 %, respectivament). Malgrat tot, la incidència en fruits avortats no va ser reduïda pels tractaments biològics, indicant que aquest teixit representa una important font d'inòcul. No obstant això l'abundància de fruits avortats és generalment reduïda i depenent de la varietat, per la qual cosa la rellevància d'aquest teixit per la PBC és variable.

La població de *C. sake* sobre la superfície de grans de raïm va ser monitoritzada durant els assajos de camp, recuperant les poblacions dels blocs tractats després i justament abans de cada aplicació. Les poblacions es van mantenir entre 5.5 i 7 Log CFU g^{-1} sobre flors i 4-6 Log CFU g^{-1} sobre baies. En les condicions de clima mediterrani de la zona de Lleida (2009 i 2010), les poblacions van decréixer 1-2 log entre aplicacions, mentre que no van decréixer en les

condicions de clima atlàntic de les vinyes de Bordeus. Aquests resultats, juntament amb els obtinguts en els assaigs amb règims climàtics simulats, van confirmar la importància de les condicions climàtiques per a la supervivència de *C. sake* a camp.

La supervivència de *C. sake* també va ser avaluada en diferents assaigs de camp i laboratori, exposant les poblacions a diferents factors abiòtics que habitualment limiten la supervivència dels ACBs, com la T, la HR, la radiació solar i la pluja. Les poblacions sobre baies es van reduir 1-3 log durant les primeres 24 hores d'exposició a condicions limitants d'alta T i baixa HR, mantenint poblacions sobre el nivell de detecció després de 72 hores d'exposició. Per tant, *C. sake* va demostrar la seva capacitat de supervivència indicant l'alt potencial d'aquest ACB per a la seva aplicació a camp. Las poblacions de *C. sake* van ser molt sensibles als factors abiòtics en el primer període després de l'aplicació (6-24 hores), mentre que un període d'establiment de les poblacions en condicions òptimes (24-48 hores; 20-22 ^{0} C i 85-100 % HR), previ a l'exposició a pluja o condicions limitants, va incrementar significativament la persistència i supervivència de *C. sake*. Aquestes dades van permetre formular recomanacions d'aplicació a camp per aquest ACB.

En conclusió, els resultats presentats en aquesta tesi doctoral demostren l'alta eficàcia dels tractaments amb *C. sake* CPA-1 i FC, que va ser similar o major que la d'altres productes registrats i en ús, confirmant la fiabilitat i el potencial d'aquests mètodes de control biològic per al control de la PBC en vinya. D'altra banda, s'han obtingut noves dades sobre la supervivència de *C. sake* i l'epidemiologia de *B. cinerea*, importants per a futurs estudis de control biològic d'aquesta malaltia. Aquesta informació també aporta importants aplicacions pràctiques per optimitzar els tractaments amb *C. sake* i altres mitjans de control alternatius als fungicides de síntesi.

Control of *Botrytis* bunch rot (*Botrytis cinerea*) by applications of *Candida sake* CPA-1 and other alternative strategies to synthetic fungicides in winegrapes

Botrytis bunch rot (BBR) of grapes, caused by *Botrytis cinerea*, is a fungal disease responsible of important economic losses in winegrowing regions, due to quantitative yield losses and the negative effect of *B. cinerea* infected berries on wine quality. The application of synthetic fungicides has been the conventional approach to control this disease in the field. However, due to the development of resistance to fungicides by the pathogen and to avoid the presence of residues in grapes and wines, the use of synthetic fungicides is being restricted and, in some cases forbidden. In addition, the control of BBR in organic viticulture is reduced to cultural practices and few products are available for growers. Therefore, in both conventional an organic viticulture, there is a lack of alternatives to manage BBR and many studies have been carried out on potentially effective treatments with Biological Control Agents (BCAs) and Natural products.

The biocontrol yeast *Candida sake* CPA-1 had demonstrated to be effective to control BBR applied in combination with Fungicover in previous studies. However, several aspects regarding *C. sake* and FC modes of action, the factors affecting *C. sake* survival and the optimisation of field applications remained unknown, whereas they represented key information for future product development and implementation by growers. Therefore, the present PhD thesis investigated these aspects including four objectives: **1)** to study and compare the efficacy of *C. sake* plus FC with other biologically-based treatments against BBR on field and laboratory experiments, evaluating their efficacy at harvest, their effect on secondary inoculum sources and their mode of action (Chapters 1, 2, 4 and 6); **2)** to test the effect of field applications of biologically-based treatments on sour rot of grapes at harvest (Chapter 3), another problematic fungal disease in hot summer climate vineyards. Moreover, **3)** the epidemiology of *B. cinerea* in Mediterranean climate vineyards (Lleida) was also studied (Chapters 2, 5), in order to determine the most important phenological stages along the season for the development of secondary inoculum sources in the bunch and the later BBR at harvest; and lastly, **4)** to investigate the effect of the abiotic factors determining the survival of *C. sake* CPA-1 after field applications, such as Temperature (T), Relative Humidity (RH), rainfall and solar radiation (Chapters 6, 7).

To study the efficacy of *C. sake* plus FC and biologically-based products, field experiments were carried out during two growing seasons in Lleida (Catalonia, Spain; 2009 and 2010) and one season, 2012, in Bordeaux (France). The evaluation of BBR at harvest in the three seasons showed that four to six applications of *C. sake* ($5x10^7$ CFU mL^{-1}) plus FC (50 g L^{-1}) along the season reduced disease incidence by 48 % to 67 % and severity by 82 % to 90 %, compared to the untreated control. Moreover, treatments consisting of two applications focused in flowering and pre bunch closure also achieved similar severity reductions. The combination of the BCA *C. sake* and FC demonstrated in general better efficacy than FC alone in both, field and laboratory studies. All the biologically-based treatments evaluated (*C. sake* CPA-1, Fungicover, chitosan, and *U. oudemansii*), as well as the strategies based on their combinations, significantly reduced BBR at harvest in all the experiments. In addition, significant reductions of sour rot severity was observed in plots treated with *U. oudemansii* (2009; 38-46 % reduction compared to

the control) and *C. sake* (2009 and 2010; 40-66 % reduction), representing the first report of biological control of this disease.

In the 2009 and 2010 seasons, the incidence of *B. cinerea* in necrotic tissues within the bunch was evaluated at veraison, comparing untreated samples and samples which had received applications of biologically-based products. Treatments with FC, *U. oudemansii* and chitosan showed significant reductions of *B. cinerea* incidence on necrotic tissues inside the bunch (43-67 % compared to control), except for the aborted fruits. These results evidenced another mechanism of action of these treatments.

Due to the observed high efficacy of FC alone, an investigation on the efficacy and the mode of action of this fatty acid-based product was carried out, comparing the results achieved with the commercialised product Protector^{HML} (PRT). When applied at the higher doses, FC significantly reduced severity of *B. cinerea* infection on leaves and berries by 44 % to 96 %, depending on the grape cultivar, *B. cinerea* isolate and inoculation method used, whereas PRT generally presented similar efficacy than FC. A multiple mode of action was hypothesized for FoodCoat, where the biofilm established by this product interferes with *B. cinerea* germination, blocks mycelial infection and protects berry wounds. PRT mode of action was similar, although it did not modify germ tube morphology and its protection of wounds was limited.

During the 2009 and 2010 growing seasons, fungicide timing experiments were carried out, treating vineyard plots with iprodione in key phenological stages along the season. The treatments including flowering applications were the most effective, suggesting that a strategy based on flowering sprays sufficiently controls BBR in the conditions tested, with the addition of another application after veraison if conditions are favourable for *B. cinerea* from pre bunch closure onwards. Moreover, samples from the fungicide experiment were used in 2010 to determine the incidence of different *B. cinerea* secondary inoculum sources, such as latent infections, necrotic tissues within the bunch and surface inoculum. The results showed that most of latent infections and the saprophytic colonisation of necrotic tissues developed during flowering. Therefore, the evaluation of both secondary inoculum sources and BBR at harvest indicated that flowering is the most important phenological stage for *B. cinerea* development in the conditions tested.

Quantitative analysis of these data evidenced positive correlation between incidence of three inoculum sources (latent infections, infection of necrotic calyptras and aborted fruits) and BBR incidence at harvest. The three variables were included in a multiple linear regression model, which represents the first publication in which several secondary inoculum sources are correlated with BBR at harvest.

Further analysis of *B. cinerea* incidence in necrotic tissues at veraison was performed on untreated samples from two organic vineyards and two seasons. The aborted fruits were the necrotic tissue presenting higher *B. cinerea* incidence (38 %) after 10 days incubation, compared to aborted flowers (19 %) and necrotic calyptras (24 %). In addition, incidence on aborted fruit was not reduced by any treatment, indicating that this necrotic tissue represents an important secondary inoculum source. However, their abundance in bunches is generally low and highly dependent on grape cultivar. Thus, the relevance of aborted fruits for BBR may be variable, whereas treatments controlled the other two necrotic tissues.

The *C. sake* populations on grape berries were monitored during all the field efficacy experiments, recovering surface populations from treated plots after and just before applications. Populations maintained between 5.5-7 log CFU g^{-1} on flowers and 4-6 log CFU g^{-1} on berries. When applied in hot dry Mediterranean summer conditions (Lleida; 2009 and 2010 seasons), populations decreased on berries 1-2 log units between applications, whereas no decrease was observed in atlantic climate vineyards (Bordeaux; 2012 season). These findings, as well as other results from experiments with simulated climatic regimes, confirmed the importance of climatic conditions for *C. sake* survival in the field.

The survival of *C. sake* was also evaluated in different laboratory and field studies, in which the yeast populations were exposed to a range of abiotic factors usually constraining BCA development in the field, such as T and RH, solar radiation and simulated rainfall. Populations on berries were reduced by 1-3 log in the first 24 hours of exposure to limiting conditions of T, RH or sunlight, although maintained detectable levels even after 72 hours of exposure to high T and low RH. Thus, *C. sake* demonstrated high survival ability in the field and under limiting conditions and abiotic factor fluctuations, indicating the high potential of this BCA for field applications. The *C. sake* populations were very sensitive to abiotic factors in the first period just after the application (6-24 hours), whereas the occurrence of an establishment period (24-48 hours; 20-22 ^{0}C and 85-100 % RH), prior to exposure to rainfall or limiting conditions, significantly increased *C. sake* survival. These findings allowed making spray recommendations to maximise persistence of BCA populations in field applications.

In conclusion, the results of the present PhD thesis demonstrated the high efficacy of *C. sake* CPA-1 plus FC treatments, which was similar or higher than other commercialised products, confirming the reliability and potential of biologically-based strategies to control BBR. In addition, new data on survival of *C. sake* and *B. cinerea* epidemiology have been obtained, important for future research on BCAs and BBR. This information also provided important practical outcomes to optimise field treatments with *C. sake* and other biologically-based products.

INTRODUCCIÓN GENERAL

INTRODUCCIÓN GENERAL

1. Antecedentes

Botrytis cinerea Pers.:Fr., es un hongo filamentoso patogénico para una amplia variedad de huéspedes, entre los que se encuentran cultivos hortícolas y frutícolas de gran importancia económica, como el tomate, la vid o varios cultivos de fruta de hueso y pepita. Las pérdidas en viticultura y fruticultura debidas a enfermedades relacionadas con *B. cinerea* se estiman en unos 2000 millones de dólares americanos anuales (Vivier y Pretorius, 2002). En viña es causante de la podredumbre gris o podredumbre por *Botrytis* (PBC), una importante enfermedad en viñedos de zonas templadas de todo el mundo, que en condiciones climáticas favorables a su desarrollo puede destruir la cosecha por completo, mientras que también puede afectar las cualidades sensoriales del vino cuando éste es elaborado a partir de racimos con un 5% de uva afectada (Ky *et al*., 2012).

El control de esta enfermedad en el viñedo es difícil debido al ciclo biológico de *B. cinerea*, que aparte de infectar tejidos verdes de la planta, se desarrolla saprofíticamente en tejidos necróticos y senescentes, dando lugar a múltiples fuentes de inóculo primario y secundario. Los métodos de control más utilizados actualmente son la aplicación de fungicidas químicos y las prácticas culturales para reducir la humedad en el viñedo. Sin embargo, *B. cinerea* ha desarrollado resistencia a numerosos compuestos químicos utilizados haciéndolos ineficientes (Latorre y Torres, 2012, Rosslenbroich y Stuebler, 2000) y la legislación para su uso es cada vez más restrictiva para evitar la presencia de residuos en vinos y uva de mesa (Reglamento (CE) N° 396/2005, plenamente aplicable a partir de 2008). Además, la viticultura ecológica es un sector en continuo crecimiento en Catalunya y en el conjunto del Estado español, para el que los métodos de control de la PBC disponibles son reducidos. Por lo tanto, en viticultura convencional son necesarios nuevos tratamientos para el control de la PBC, que puedan ser utilizados complementariamente a los tratamientos fungicidas de principio de campaña o sustituir completamente a los tratamientos químicos, mientras que en viticultura ecológica se necesitan tratamientos para lograr un control consistente de la enfermedad a lo largo de toda la campaña.

Entre las estrategias alternativas al uso de fungicidas, la aplicación de microorganismos como agentes de control biológico (ACBs) es una de las que muestran un mayor potencial, tanto contra la PBC del racimo como contra otras enfermedades fúngicas de cultivos frutícolas (Nicot, 2011). El control biológico empleando microorganismos ha sido ampliamente estudiado por grupos de investigación de todo el mundo. En concreto, el grupo de Patología de la Post-cosecha del centro IRTA-Lleida y de la Universidad de Lleida tiene más de 20 años de experiencia en el control de las principales enfermedades de fruta de hueso y pepita. La levadura *Candida sake* CPA-1 fue aislada y desarrollada por este grupo, debido a la efectividad mostrada en el control de varias enfermedades post-cosecha de pera y manzana, incluyendo *B. cinerea*. Las características ecofisiológicas de este microorganismo y su efectividad en el control de *B. cinerea*, también en uva, sugirió la posibilidad de su aplicación en campo sobre uva

de vinificación, que se llevó a cabo durante dos campañas vitícolas consecutivas en el área de Lleida (Cañamás *et al.*, 2011), mostrando reducciones significativas de la PBC en cosecha.

El potencial mostrado por este microorganismo, así como la progresión de la viticultura ecológica y la falta de tratamientos alternativos para el control de la PBC, estimularon la investigación sobre la efectividad de tratamientos biológicos en un proyecto cuyos resultados se describen en la presente tesis doctoral. A continuación se exponen de forma más detallada las características de esta enfermedad y de los métodos evaluados para su control.

2. La podredumbre gris del racimo (*B. cinerea*)

2.1. El patógeno B. cinerea

B. cinerea es un hongo filamentoso de la familia Sclerotinicaeae, capaz de infectar a más de 230 especies de plantas huéspedes (Elad *et al.*, 2004b). Como todo los miembros del género *Botrytis* actúa como patógeno necrotrofo, infectando los tejidos del huésped e induciendo su necrosis, pero además es capaz de sobrevivir y formar estructuras de resistencia (esclerocios) en el tejido necrótico generado por la infección (van Kan, 2006). De esta manera, las fuentes de inóculo en el cultivo son múltiples, dada su posibilidad de infectar y sobrevivir en partes verdes y muertas del propio cultivo y de las malas hierbas adyacentes (Holz *et al.*, 2004). La Fig. 1 resume el ciclo del patógeno, el proceso de infección y algunas de las formas biológicas en las que se puede encontrar.

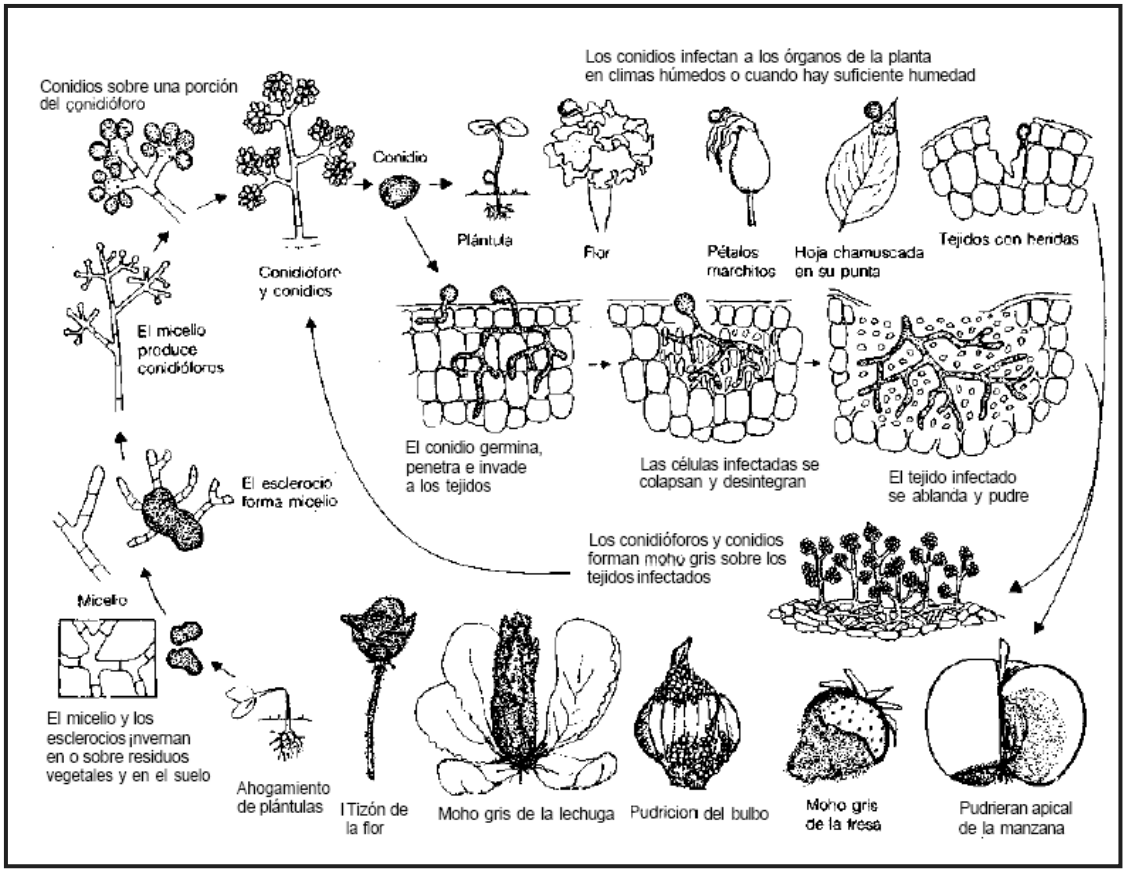


Figura 1 Ciclo biológico general y desarrollo del proceso infectivo de *Botrytis* spp. (Agrios, 1995)

El hongo puede existir en estos diferentes hábitats como micelio, micro y macro conidias (Fig. 2), clamidosporas, esclerocios, apotecios y ascosporas, dispersándose por diferentes medios, mientras que la dispersión depende de que se den condiciones favorables para su crecimiento y esporulación, principalmente humedad en superficie y temperatura (T) (Jarvis, 1980).

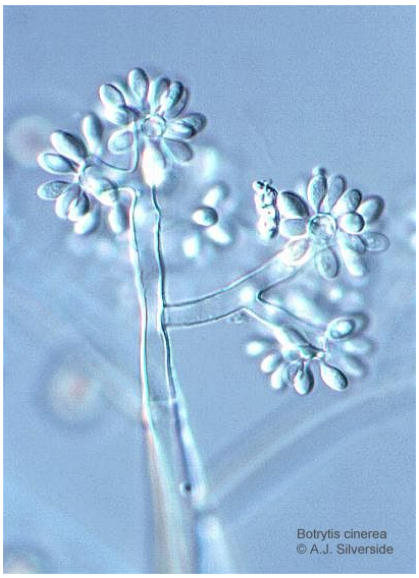


Figura 2 Microfotografía de un conidióforo de *B. cinerea*. Pueden apreciarse las conidias y la estructura arbuscular del conidióforo

Las conidias son transportadas mayoritariamente por el viento o por los diversos insectos que pueden actuar como vectores de dispersión: mosca del vinagre (*Drosophila melanogaster*) (Louis *et al.*, 1996), polilla del racimo (*Lobesia botrana*) (Fermaud y Lemenn, 1989), trips de flores de Nueva Zelanda (*Thrips obscuratus*) (Fermaud y Gaunt, 1995) y mosca de la fruta (*Ceratitis capitata*) (Engelbrecht *et al.*, 2004). Una vez que las conidias llegan a la superficie susceptible del huésped, la formación del tubo germinativo comienza tras 1-3 horas en presencia de agua, dando lugar a apresorios simples y/o compuestos aproximadamente a las 6 horas (Fourie y Holz, 1994, 1995). También pueden formarse apresorios multicelulares y lobulados, especialmente con la adición de nutrientes exógenos (Shirane y Watanabe, 1985), aunque se ha observado su formación sobre hojas y bayas de viña sin necesidad de estos nutrientes (Coertze *et al.*, 2001). El apresorio puede dar lugar a penetración directa a través de la epidermis del tejido, aunque sobre la superficie de uvas no siempre ocurre y depende del grosor de la epidermis, característico de cada variedad. Sin embargo, en las enfermedades causadas por *Botrytis* spp. el papel de las conidias parece tener una menor importancia que el de la infección por propágulos de micelio o de tejidos necróticos colonizados saprofíticamente (Holz *et al.*, 2004). La penetración, por tanto, puede ocurrir a través de tejidos completamente sanos y aberturas naturales como estomas (Fourie y Holz, 1995) o heridas (Coertze y Holz, 2002).

Otra vía de penetración es la entrada a través de órganos especializados de la planta, como glándulas secretoras y órganos florales (pistilo, estambres, unión entre sépalos), que suelen ser el origen de las infecciones latentes. Estas infecciones son especialmente importantes como fuente de inóculo secundario, que puede dar lugar a infecciones en el periodo pre-cosecha a medida que la madurez de los frutos avanza y las defensas de la planta disminuyen (Pezet *et*

al., 2003) o en el periodo post-cosecha, provocando cuantiosas pérdidas dada la imposibilidad de ser detectadas.

Las bases moleculares del proceso infectivo de *B. cinerea* sobre los tejidos del huésped han sido ampliamente estudiadas en los últimos años y revisadas en varias publicaciones (Elad *et al.*, 2004a, van Kan, 2006, Williamson *et al.*, 2007). La investigación en las relaciones huésped-patógeno de *Botrytis* spp. y sus huéspedes avanza continuamente dada la importancia económica de la enfermedad y a su consideración como patógeno necrotrofo de referencia. Algunos de los más recientes avances han sido expuestos en las comunicaciones del XVII Simposium de *Botrytis*, celebrado en Bari, Italia, en Junio de 2013 (http://www.xvibotrytisymposiumbari.it/files/Programme%20XVI%20International%20Botrytis%20Symposium.pdf; Acceso 25/09/2013).

2.2. Ciclo epidémico de B. cinerea en viña

Dada la multiplicidad de plantas huéspedes susceptibles de infección y la variedad de formas biológicas de *B. cinerea*, comentadas anteriormente, el ciclo biológico del patógeno en el viñedo es relativamente complejo (Fig. 3).

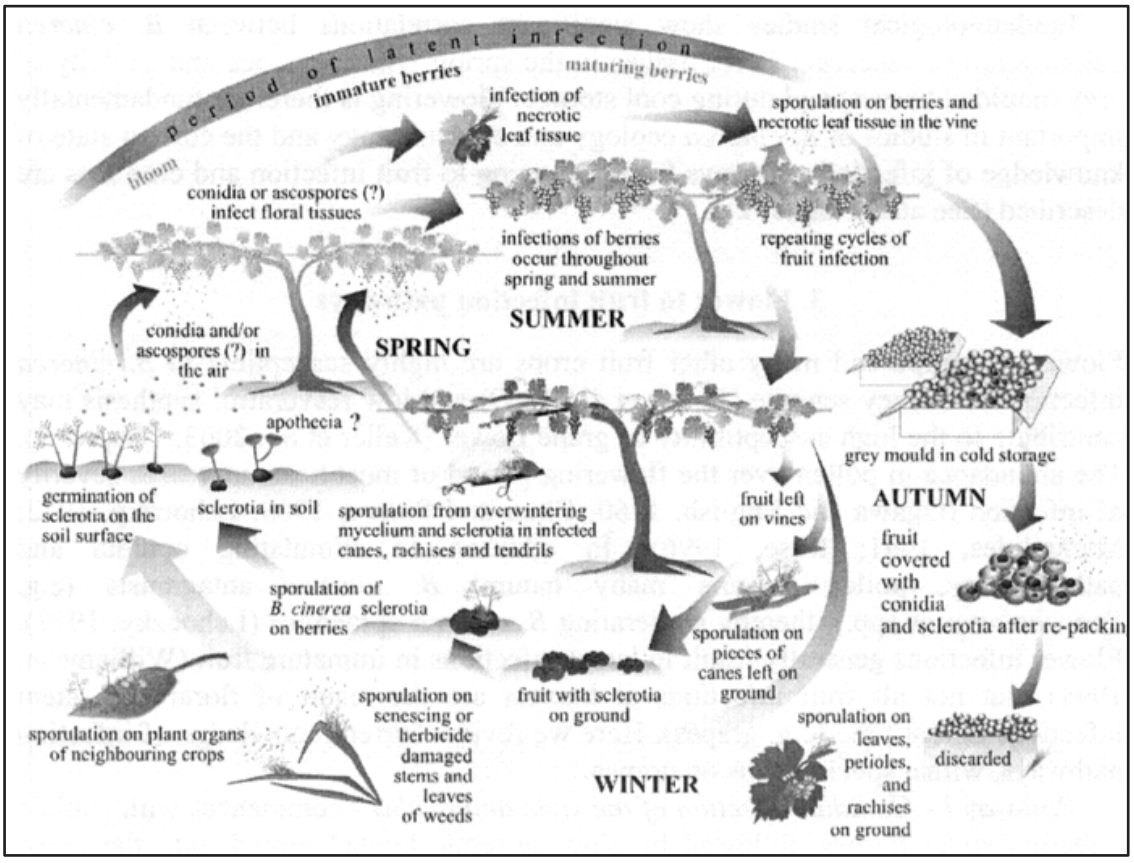


Figura 3 Ciclo biológico de *B. cinerea* y desarrollo de la podredumbre gris en uva de mesa y vinificación (Elmer y Michailides, 2004)

El inóculo primario se produce a partir de la esporulación de micelio o esclerocios que han pasado el invierno en el material necrótico de la viña o de las malas hierbas depositadas sobre el suelo, que han sido correlacionados con una mayor incidencia de infecciones de flores (Nair *et al.*, 1995). La cuantificación de diferentes elementos de este material necrótico en el momento de floración, realizada en diferentes estudios en Nueva Zelanda, indicó que los restos de racimos, peciolos de hojas, zarcillos y ramas eran las fuentes de inóculo más importantes (Jaspers *et al.*, 2013, Mundy *et al.*, 2012, Seyb, 2004).

Durante el periodo de floración, el inóculo primario es abundante, mientras que las flores son altamente sensibles a la infección (Jersch *et al.*, 1989, Latorre *et al.*, 2002a, Nair y Allen, 1993). Estas infecciones de flores generalmente no se desarrollan más y permanecen latentes, siendo el origen de varias de las vías de infección de las bayas a partir del momento de envero. Estas vías de infección fueron detalladamente descritas por Elmer y Michailides (2004) y consisten en: *Vía 1*: Infección por conidias del pistilo y los ovarios; *Vía 2a*: Infección por conidias de estambres y/o pétalos; *Vía 2b*: Infección del fruto a través del pedicelo del fruto; *Vía 3*: Infección por conidias y colonización saprofítica de restos florales; *Vía 4*: Acumulación de conidias en el racimo en desarrollo; *Vía 5*: Infección por conidias del fruto en maduración; *Vía 6*: Acumulación de conidias y dispersión hasta las heridas de recolección (descrita para kiwi).

Además de estas vías, en el caso de la PBC en viña, es especialmente importante el papel de la polilla del racimo, que ha demostrado arrastrar conidias y propágulos de *B. cinerea* pegadas a la superficie del organismo, las cuales son depositadas en el interior de las bayas cuando la larva se desplaza por el interior de las mismas alimentándose (Fermaud y Lemenn, 1989, Fermaud y Giboulot, 1992, Fermaud, 1998, Mondy *et al.*, 1998). Este inóculo de *B. cinerea* produce infecciones que pueden extenderse al resto del racimo incrementando la severidad en el momento de cosecha, especialmente relacionado con la tercera generación de *L. botrana* según el reciente estudio de Pavan *et al.*, (en prensa).

A partir de envero, la susceptibilidad de las bayas aumenta debido a la reducción de compuestos fenólicos, taninos, pectinas y el aumento de azúcares durante el proceso de maduración (Goetz *et al.*, 1999, Pezet *et al.*, 2003), notablemente en la epidermis de las bayas (Deytieux-Belleau *et al.*, 2009), favoreciendo el proceso de infección. Es en este periodo post-envero cuando la infección de las bayas va progresando y extendiéndose también de unas bayas a otras en el racimo y dando lugar a los síntomas característicos de la enfermedad: ligera descomposición de la pulpa y cambio de color de las bayas infectadas, hacia un tono marrón violáceo en variedades blancas y hacia tonos rojizos en variedades tintas (Fig. 4)

La importancia de las diferentes vías de infección y fuentes de inóculo secundario en el desarrollo epidémico post-envero, ha sido demostrado en diferentes estudios, sobre todo para algunas de las más importantes como las infecciones latentes (Chebil *et al.*, 2003, Keller *et al.*, 2003, Pezet *et al.*, 2003, Sanzani *et al.*, 2012) o tejidos necróticos colonizados saprofíticamente (Seyb, 2004, Wolf *et al.*, 1997). Sin embargo, su contribución a la severidad en el momento de cosecha es variable y varios aspectos relativos al desarrollo del inóculo secundario y su traducción en infecciones de bayas no son comprendidos completamente.

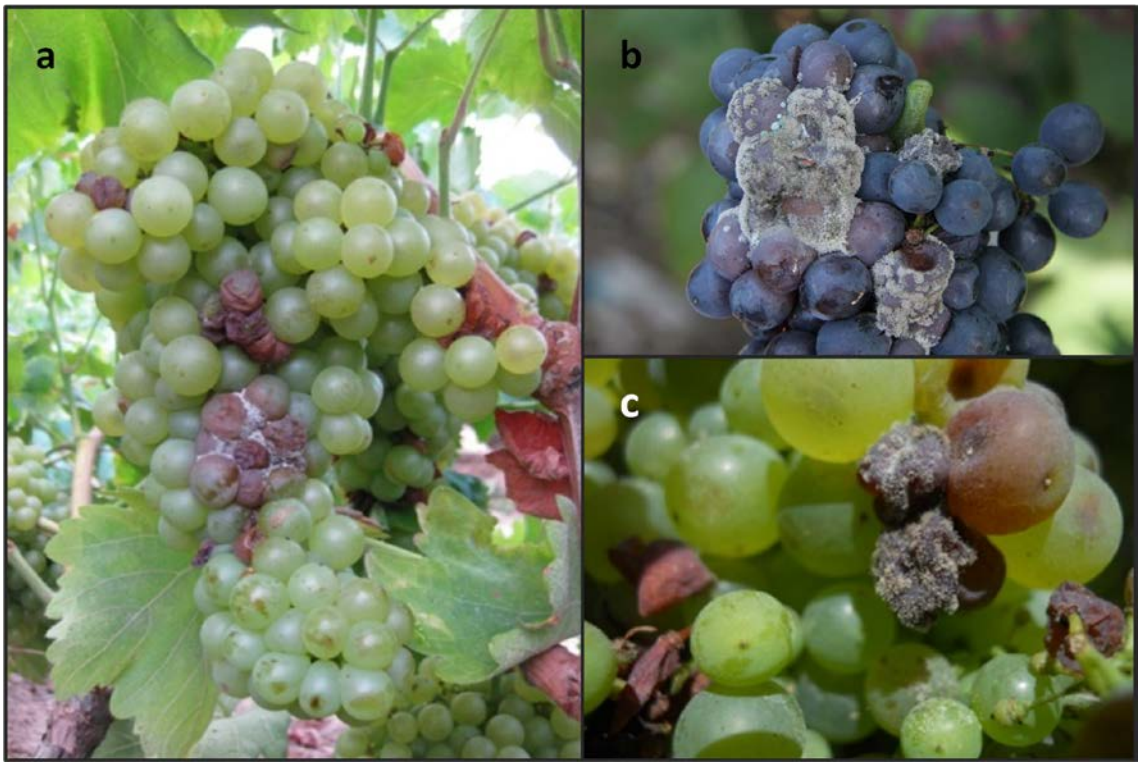


Figura 4 Sintomas de la podredumbre por *B. cinerea* en campo. a) Racimo variedad 'Macabeu' con varias partes infectadas, puede observarse el tono marrón que adquieren las bayas, la gran compactación del racimo y la facilidad de transmisión de la enfermedad dentro del racimo; b) síntomas sobre la variedad tinta Tempranillo, con abundante esporulación y las bayas tomando un tono más rojizo; c) esporulación sobre bayas infectadas y parcialmente desecadas, transmitiendo la infección por contacto a las bayas adyacentes.

2.3. Factores que determinan la PBC en cosecha

Los factores que determinan un mayor o menor desarrollo de la enfermedad en campo, en cuanto al inóculo secundario y la posterior podredumbre, son básicamente tres: susceptibilidad del huésped, virulencia del aislado de *B. cinerea* y las condiciones meteorológicas.

La susceptibilidad a la infección es altamente variable en función de la variedad de uva, pero las diferencias parecen responder más bien a una epidermis más gruesa, mayor contenido en ceras y menor cantidad de poros, que a una resistencia antifúngica constitutiva de los tejidos de cada variedad (Mlikota *et al.*, 2003). Por tanto, la estructura de la epidermis parece ser un factor determinante en la resistencia ontogénica de las uvas a la infección por *B. cinerea* (Deytieux-Belleau *et al.*, 2009), que actúa como primera barrera física contra la infección. Sin embargo, otros factores propios de la viña son también determinantes para su susceptibilidad, como la compactación de los racimos, que es muy dependiente de la variedad (Alonso-Villaverde *et al.*, 2008, Hed *et al.*, 2009, Valdés-Gómez *et al.*, 2008). El vigor de la planta y la cantidad de aporte de nitrógeno al cultivo, están también directamente relacionados con la susceptibilidad (Deytieux-Belleau *et al.*, 2009, Keller *et al.*, 2001). Por el contrario, el calcio parece incrementar los niveles de celulosa y pectinas en el huésped y muestra un efecto inhibidor sobre *B. cinerea* (Elmer y Michailides, 2004). Un factor relacionado con la susceptibilidad del huésped sería también la presencia de heridas, que representa una de las principales vías de acceso del

patógeno a los tejidos de la planta, ya que supone la eliminación de las barreras físicas establecidas por el huésped. En el caso de uva, estas heridas pueden producirse de forma natural principalmente en la base del pedicelo, a causa de la presión ejercida por el contacto entre bayas después del cierre del racimo, mientras que también pueden producirse microfisuras (*'berry splitting'*) en la superficie de las bayas debido a una irrigación excesiva (lluvia o irrigación artificial). Otros agentes causales de heridas con especial importancia en viña son los ataques de la polilla del racimo (*L. botrana*), las granizadas y las heridas provocadas en la manipulación del viñedo aplicando técnicas culturales como el deshojado (especialmente deshojado mecánico).

En cuanto a la virulencia del aislado, se han caracterizado cepas en regiones vitícolas de todo el mundo con virulencia que puede variar desde casi avirulentas hasta altamente agresivas, dado que se trata de una especie con enorme variabilidad genética, que cambia entre generaciones y en condiciones de campo e *in vitro*, debido a la formación de conidias multinucleares e hifas compartimentadas (Beever y Weeds, 2004, Elad *et al.*, 2004b). Dos subgrupos han sido caracterizados en función de la presencia de diferentes transposones (*vacuma* y *transposa*), los cuales presentan diferentes grados de crecimiento micelial y virulencia (Martinez *et al.*, 2003, Martinez *et al.*, 2005). Sin embargo, la presencia en el viñedo de diferentes aislados y subgrupos es variable y éstos suelen estar mezclados y en continuo flujo entre regiones, haciendo que a efectos prácticos en situaciones de campo, este factor no sea muy determinante para la PBC en cosecha.

Las condiciones meteorológicas afectan en gran manera la PBC ya que determinan el microclima en el interior del viñedo. Las condiciones climáticas y microclimáticas fueron el principal factor de riesgo de infección en el citado estudio de Deytieux-Belleau *et al.* (2009) que evaluó la correlación de casi 40 variables con la incidencia y severidad de la PBC en cosecha, identificando cinco variables climáticas como las más importantes (precipitación, potencial de evapotranspiración, potencial hídrico previo al amanecer, Humedad Relativa (HR) > 90% en el viñedo, T > 30 °C). Igualmente, la actividad de agua (a_ω) fue el factor más determinante para la infección de *B. cinerea* en el mismo estudio.

Estos datos subrayan la importancia de la meteorología en el desarrollo de la enfermedad, que es muy elevado cuando las condiciones ofrecen disponibilidad de agua y temperaturas similares al óptimo de crecimiento *in vitro* (20-22 °C), en las que la capacidad de infección de *B. cinerea* en flores y bayas es mayor (Latorre *et al.*, 2002a, Nair y Allen, 1993). Las condiciones descritas de alta humedad relativa y temperaturas moderadas con máximas por debajo de los 30 °C son las que se producen habitualmente en zonas de clima templado-atlántico, en las cuales el riesgo epidémico y la incidencia y severidad de la PBC es mayor de forma general. En zonas de clima mediterráneo, el riesgo epidémico es más variable, dependiendo de que las condiciones específicas de la campaña sean favorables para el patógeno, así como de efectos microclimáticos que pueden reducirse a pequeños enclaves o pocas parcelas, haciendo la epidemiología de la enfermedad más específica de cada zona.

2.4. La PBC y otras podredumbres del racimo

Además de PBC, que es la podredumbre del racimo más importante por su abundancia, su ubicuidad y su capacidad destructiva, existen otras podredumbres fúngicas que pueden afectar la producción en el viñedo. Las podredumbres oportunistas infectan las bayas de forma indirecta, aprovechando heridas o aberturas presentes en la epidermis. Los microorganismos que habitualmente dan lugar a estas enfermedades son hongos como *Aspergillus*spp., *Alternaria* spp., *Rhizopus*spp., *Cladosporium herbarum*, *Penicillium* spp. (Ministerio de Agricultura y Mundi-Prensa, 2004). En otras regiones vitícolas de clima subtropical como el Hunter Valley de Australia por ejemplo, se pueden encontrar epidemias severas de *ripe rot* (*Colletotrichum acutatum*) y *bitter rot* (*Greeneria uvicola*). Sin embargo, la más importante de estas otras enfermedades es la podredumbre ácida.

La etiología de esta enfermedad es controvertida, ya que no existe un patógeno concreto responsable de la enfermedad, aunque actualmente está aceptado que se trata de un complejo de levaduras y bacterias capaz de realizar reacciones fermentativas en bayas tras penetrar en ellas de forma oportunista (Barata *et al.*, 2012a, Gravot *et al.*, 2001, Guerzoni y Marchetti, 1987, Huber *et al.*, 2011, Oriolani *et al.*, 2009, Wei *et al.*, 2011). La sintomatología se caracteriza por el color marrón que toman las bayas, la pérdida de turgencia por la desintegración de los tejidos internos (Fig. 5) y por el intenso olor a vinagre que desprenden (Gravot *et al.*, 2001, Guerzoni y Marchetti, 1987). Así mismo, la mosca del vinagre *D. melanogaster* actúa como uno de los principales vectores de transmisión (Barata *et al.*, 2012b, Fermaud *et al.*, 2000).

Figura 5 Sintomas de la podredumbre ácida sobre racimo de variedad 'Macabeu'. Las bayas toman un tono marrón pálido, la pulpa se desintegra dando lugar a un líquido con fuerte olor a vinagre. Este producto de la fermentación las bayas cae sobre el racimo, lo que favorece la infección de las partes afectadas y atrae a la mosca del vinagre *D. melanogaster*, vector de transmisión de la enfermedad.

Se trata de una enfermedad con efectos muy perjudiciales para la calidad sensorial en la vinificación (Steel *et al.*, 2013). Actualmente esta enfermedad está tomando mayor importancia económica en diferentes regiones vitícolas como Italia (Nigro *et al.*, 2006), Argentina (Nally *et al.*, 2013, Oriolani *et al.*, 2009), China (Wei *et al.*, 2011), noreste de EE.UU. (Niagara región) (Huber *et al.*, 2011) o California (Joe Smilanick, USDA, comunicación personal). Sin embargo, no existen mecanismos efectivos de control, ni químicos ni biológicos, con la excepción de la moderada eficacia mostrada por tratamientos con sales minerales (Nigro *et al.*, 2006), prácticas culturales (Rooney-Latham *et al.*, 2007) o los ensayos *in vivo* recientemente publicados apuntando el control biológico con levaduras (Nally *et al.*, 2013).

Tanto la podredumbre ácida, como el resto de podredumbres mencionadas, suelen ocurrir simultáneamente con la PBC en el viñedo. Sin embargo, la prevalencia de la PBC respecto a las demás suele estar muy condicionada también por las condiciones climáticas, con comportamiento irregular dependiendo de la campaña (Ministerio de Agricultura y Mundi-Prensa, 2004). Por ejemplo, las temperaturas mayores de 30 °C favorecen la *ripe rot* y la *bitter rot* respecto a la PBC en ensayos de campo y laboratorio (Steel y Greer, 2008, Steel *et al.*, 2010, Steel *et al.*, 2011), mientras que las temperaturas altas parecen favorecer también el desarrollo de la podredumbre ácida (Galet, 1999). Sin embargo, los factores más determinantes para la prevalencia de las distintas enfermedades y el mecanismo subyacente son en gran parte desconocidos.

3. Métodos de control de la PBC en viña

Las diferentes estrategias utilizadas para el control de la PBC y su efecto respecto a los factores que favorecen la enfermedad se presenta resumida gráficamente en la Fig. 6. En ella se interrelacionan distintos factores propios de la planta que determinan su susceptibilidad (vigor, mecanismos de defensa) con factores bióticos (insectos y otros animales) y abióticos (humedad, accidentes meteorológicos) habituales en el viñedo. Así mismo, se enumeran las distintas técnicas culturales y tipos de productos que pueden aplicarse para el control de la PBC, indicando los elementos con los que interaccionan.

3.1. Prácticas culturales

Según Nair *et al*. (1995), en el proceso de infección floral el factor más determinante es la cantidad de inóculo primario presente, mientras que el factor más importante para el desarrollo del inóculo secundario a partir de envero, son las condiciones meteorológicas. Por lo tanto, las prácticas culturales suelen estar enfocadas a: 1) la reducción de fuentes de inóculo, sobre todo primario para evitar la formación de inóculo secundario y 2) la reducción de la humedad presente en el viñedo durante el desarrollo de los racimos.

En el primer caso, la eliminación de fuentes de inóculo secundario puede hacerse de forma manual, aunque debido al coste del trabajo que supone, también se ha investigado su regulación mediante el uso de acolchados (Jacometti *et al.*, 2007, 2010, Mundy y Agnew, 2002). Así mismo, también se ha evaluado la reducción de fuentes de inóculo secundario en tejidos necróticos mediante la aplicación de chorros de aire (Wolf *et al.*, 1997).

Para la reducción de la humedad, las técnicas más utilizadas son el deshojado parcial (Cravero *et al.*, 2004, Evers *et al.*, 2010, Mehofer *et al.*, 2009, Molitor *et al.*, 2011, Stapleton y Grant, 1992, Zoecklein *et al.*, 1992), que ha mostrado importantes reducciones de la enfermedad en estos estudios y es una práctica habitual en muchas regiones vitícolas.

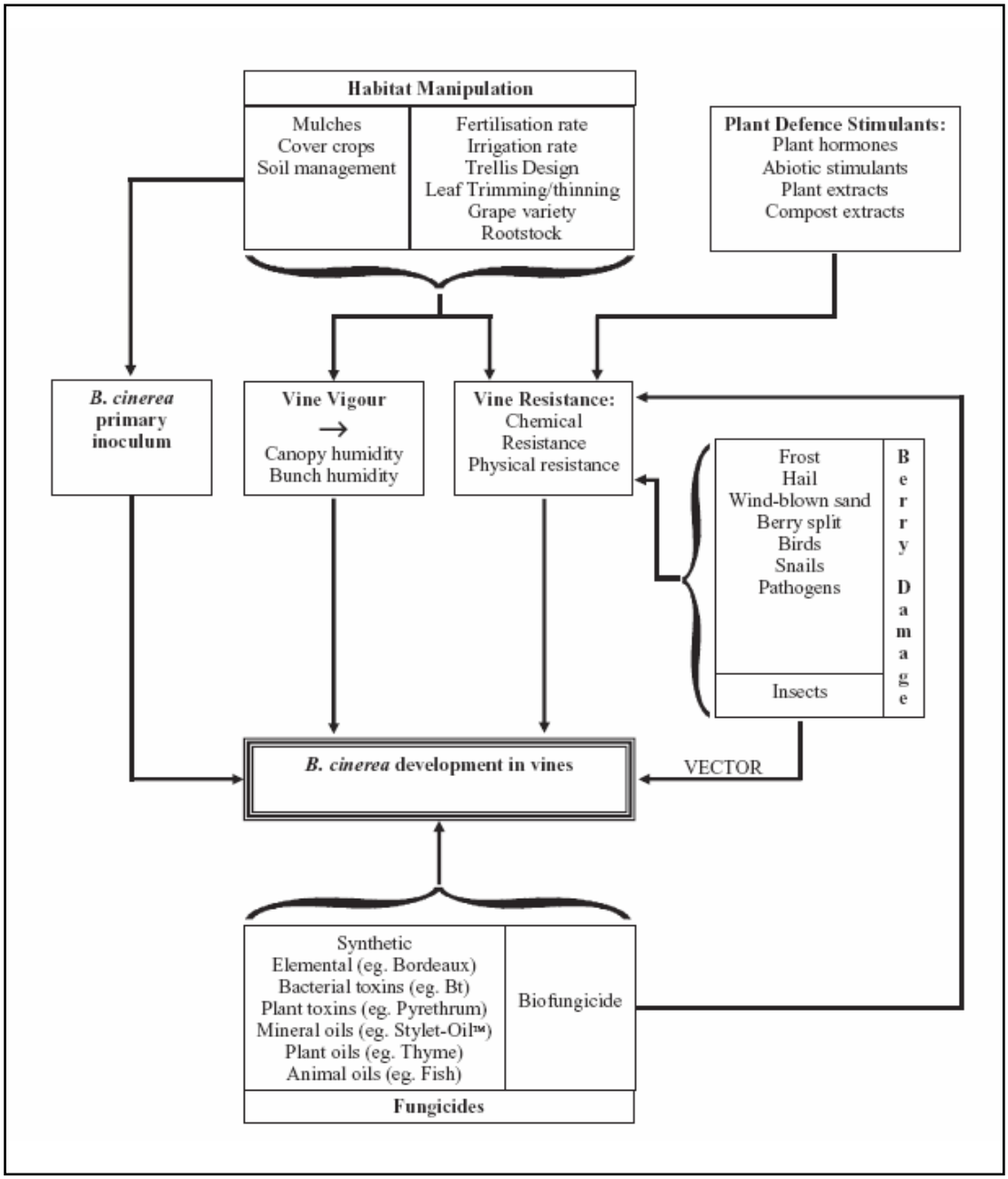


Figura 6 Métodos de control de *B. cinerea* en viña y factores que afectan su efectividad en campo (Jacometti *et al.* 2010)

Otros trabajos han evaluado el uso de prácticas para reducir el vigor de la viña, reduciendo la compactación del racimo y mejorando el microclima en su interior. Por ejemplo, mediante el uso de portainjertos de reducido vigor o la aplicación de un déficit hídrico (Sicher *et al.*, 1995, Valdés-Gómez *et al.*, 2008), o mediante la reducción mecánica de la carga de cosecha mediante podas y descarga manual de los racimos (Hanni *et al.*, 2013, Spring y Viret, 2011, Tardaguila *et al.*, 2012).

Además, añadiremos aquí el control de la polilla del racimo (*L. botrana*), ya que no se trata de un control directo sobre *B. cinerea*, sino sobre uno de sus principales vectores. Este lepidóptero realiza entre 2 y 4 generaciones en el viñedo, las últimas de las cuales coinciden con

el periodo cercano a cosecha. La generación más importante epidemiológicamente es la tercera (Fermaud y Giboulot, 1992, Fermaud y Lemenn, 1992), y los resultados del reciente estudio de (Pavan *et al.*, en prensa) señalan la ineficacia de los tratamientos que afectan a la segunda generación. Para el control de *L. botrana* actualmente se dispone de métodos como trampas de confusión sexual, o el control biológico con *Bacillus thuringiensis.* Existen numerosas formulaciones de este ACB en el mercado, es ampliamente utilizado en horticultura y también en viña en viticultura ecológica, y resulta especialmente efectivo si la aplicación se realiza en los estados iniciales del desarrollo larvario. También existen numerosos insecticidas químicos, los cuales han sido compilados por la agencia estatal norteamericana University of California Cooperative Extension http://cenapa.ucanr.edu/newsletters/UPDATED_insecticide_list_for_Lobesia_botrana25609.pdf ; Acceso 24 Septiembre 2013).

3.2. Control químico

La aplicación de fungicidas químicos de síntesis es la estrategia de control más extendida actualmente. Se trata de moléculas químicas tóxicas para el patógeno o que inhiben su crecimiento alterando distintas rutas metabólicas o procesos fisiológicos del organismo (Lerroux, 2004). Las diferentes familias de antibotríticos y sus modos de acción están extensamente detallados en los trabajos de Rosslenbroch y Stuebler (2000) y Leroux (2004).

En viña, el control con fungicidas de síntesis se ajusta a un calendario de aplicaciones, que suele realizarse en base los estados fenológicos. El sistema fenológico clásico, desarrollado en Francia en los años 60, incluye aplicaciones en cuatro estados fenológicos clave: A- Fin de la floración; B – Pre-cierre de racimo; C – Principio de envero; D – 3-4 semanas antes de la fecha de vendimia (Galet, 1999), mientras que las recomendaciones de las agencias de extensión agraria y otros textos se basan en adaptaciones de este sistema (Gil, 2003, Servei de Sanitat Vegetal, 2002). Otros sistemas, desarrollados a partir de los años 70 y 80 del siglo pasado se basan en reglas de decisión sencillas en base a periodos de humectación foliar o al estado de potencial de infección (Galet, 1999). En cualquier caso, los sistemas de decisión no son fácilmente accesibles y, dependiendo de la región vitícola, las prácticas habituales de los viticultores varían entre aplicaciones puntuales hasta aplicaciones periódicas durante buena parte del periodo post envero, dependiendo de la decisión personal del agricultor, ya que estas prácticas reciben un escaso y difícil control por parte de las instituciones.

El control químico es efectivo en la mayoría de los casos, consiguiendo elevadas reducciones de la podredumbre en cosecha, aunque también puede ser variable o ineficaz, dependiendo de las condiciones meteorológicas y de una correcta aplicación del producto en momentos clave del desarrollo de la enfermedad. Sin embargo, el uso de fungicidas presenta una serie de importantes inconvenientes. Por un lado, *B. cinerea* ha sido capaz de desarrollar resistencia a gran parte de las moléculas de síntesis aplicadas (Latorre *et al.*, 2002b, Leroux, 2004, Petit *et al.*, 2010, Rosslenbroich y Stuebler, 2000), dando lugar incluso a fenotipos multirresistentes (Grosman *et al.*, 2012, Latorre y Torres, 2012, Leroch *et al.*, 2011, Yan *et al.*, 2012, Zhao *et al.*, 2010) y generando problemas de resistencia a nivel regional muy amplio (Walker *et al.*, en prensa).

Por otro lado, los efectos nocivos de algunos fungicidas para el medio ambiente (Komárek *et al*., 2010) y la salud humana, de los pesticidas químicos en general (Bretveld *et al*., 2007, Farquhar *et al*., 2009, Younglai *et al*., 2007), han supuesto la regulación de su uso por parte de los estados y han provocado rechazo por parte de los consumidores (Crane *et al*., 2006). Por este motivo, actualmente existen en muchos países legislaciones restrictivas para el uso de estos compuestos y para los niveles de residuos permitidos sobre el producto final de la mayoría de cultivos. Además, el uso de fungicidas y sus residuos pueden alterar la calidad organoléptica del vino elaborado posteriormente (Cus *et al*., 2010, Darriet *et al*., 2001, Gonzalez-Alvarez *et al*., 2012, Oliva *et al*., 1999, Oliva *et al*., 2008).

De esta forma, la reducción del uso de fungicidas se ha convertido en un objetivo a cumplir en muchos países con especial énfasis en los últimos años, para conseguir una mayor calidad del producto, promover un sistema de producción menos dañino con el medio ambiente y que no implique riesgos para la salud, además de para reducir los costes de producción del vino.

Dentro de la perspectiva del control químico, la reducción de los fungicidas pasa por un uso más racional que maximice la efectividad de las aplicaciones y limitar su número al mínimo. Para ello se han desarrollado distintas herramientas de decisión en base a modelos de predicción del riesgo epidémico, que han sido desarrollados gracias al mayor conocimiento de la epidemiología del patógeno y a la monitorización de los factores meteorológicos determinantes para su desarrollo. Actualmente se cuenta con varios sistemas de este tipo desarrollados en Francia (Fermaud *et al*., 2003, Fermaud *et al*., 2010), Chile (Broome *et al*., 1995), Nueva Zelanda (Agnew *et al*., 2004), mientras que en el Estado español se cuenta con el sistema DOSAVIÑA (Gil *et al*., 2011) o adaptaciones de otros sistemas (Diez, 2010, Urbaso, 2003), además de otros sistemas en desarrollo, investigados por diferentes grupos (Aira *et al*., 2009, Diez-Navajas y Ortiz-Barredo, 2011).

3.3. *Métodos alternativos*

Debido a los inconvenientes y restricciones del uso de moléculas de síntesis, actualmente existe una necesidad evidente de productos que puedan ser utilizados de forma complementaria a la aplicación de fungicidas, especialmente a partir de envero, cuando el riesgo de aparición de residuos es mayor. Así mismo, existe la voluntad por parte de muchos viticultores de sustituir el uso de fungicidas en lo posible para reducir al máximo su efecto sobre el medio ambiente en el viñedo, aunque no realicen un manejo del cultivo de tipo ecológico. Del mismo modo, la necesidad es aún mayor en viticultura ecológica, un sector en continuo crecimiento en el que la aplicación de pesticidas químicos no está permitida. El control de la PBC en viticultura ecológica reside en la aplicación de técnicas culturales como las ya comentadas, pero con un especial énfasis en el manejo de la biodiversidad en el viñedo. Esto puede conseguirse mediante el mantenimiento de bordes con alta riqueza de especies y de cubiertas vegetales, que además regulan el vigor de la planta (Jacometti *et al*., 2010). Sin embargo, estos métodos de control no son siempre lo suficientemente efectivos y se necesitan tratamientos complementarios para mejorar las defensas de las plantas o suprimir el desarrollo de la PBC hasta límites aceptables.

En todas las circunstancias descritas, por tanto, existe una clara necesidad de métodos de control que no posean los inconvenientes de los fungicidas químicos y que, en el caso de la uva de vinificación, no alteren la calidad del vino. Esta necesidad ha incentivado la investigación por parte de grupos de investigación de todo el mundo en los últimos 20 años. La variedad de métodos alternativos accesibles o en desarrollo actualmente ha sido revisada en dos interesantes publicaciones de Elmer y Reglinski (2006) y Jacometti *et al*. (2010), en las que la aplicación de productos naturales (PNs) y el control biológico se consideran como algunas de las estrategias con mayor potencial para el control de la PBC en campo y son el objeto de estudio de la presente tesis, por lo que serán descritas más en detalle en las siguientes secciones de la introducción.

El conjunto de métodos de control no químico comentados, incluyendo el control biológico y los PNs, muestran en ocasiones porcentajes de reducción por debajo del límite aceptable, o su eficacia puede ser variable a causa de los factores fluctuantes de las condiciones de campo. Especialmente en el control de la PBC, debido a la complejidad del ciclo del patógeno en el viñedo, esta variabilidad puede ser grande. La combinación de varios de estos métodos de control es considerada una estrategia efectiva para superar esta variabilidad (Elmer y Reglinski, 2006), con la intención de sumar modos de acción que actúen en diferentes etapas del ciclo epidémico y sobre diferentes vías de infección.

4. El control biológico

4.1. *El control biológico con microorganismos*

El uso de microorganismos antagonistas ha sido estudiado por grupos de investigación de todo el mundo y representa una de las estrategias con mayor potencial para el control de enfermedades fúngicas (Droby *et al*., 2009, Nicot, 2011). La mayoría de estudios se han realizado en condiciones de post-cosecha, ya que resulta muy interesante para este sector debido a la prohibición del uso de fungicidas en algunos casos, a las condiciones ambientales controladas durante la conservación y la facilidad de aplicación en las centrales de fruta y hortalizas. Sin embargo, también se han llevado a cabo aplicaciones en pre-cosecha para el control de patologías de post-cosecha (Teixidó *et al*., 2010) o para el control de patologías en cultivos sin conservación post-cosecha, como la uva de vinificación (Elmer y Reglinski, 2006).

El tipo de microorganismos utilizados es muy amplio e incluye hongos filamentosos, levaduras, hongos tipo levadura ('*yeast-like fungi*') y bacterias. Los modos de acción pueden ser: antibiosis, inducción de resistencia en el huésped, competencia por nutrientes y/o espacio, parasitismo, adherencia a la superficie del patógeno, reducción de la patogenicidad del patógeno y supresión de la formación de inóculo (Elad y Stewart, 2004, Spadaro y Gullino, 2004, Teixidó *et al*., 2011). El proceso de desarrollo de un ACB es largo y complejo, comienza con la selección de varias cepas candidatas, que deben pasar numerosas pruebas de efectividad en laboratorio, adaptabilidad a condiciones de aplicación comercial o en campo, optimización de la producción, desarrollo de formulaciones estables para su comercialización, optimización de los tratamientos y formulación para su aplicación, estudios de mercado, riesgos ambientales y sanitarios, además del proceso de registro y otros aspectos regulatorios (Köhl *et al*., 2011, Teixidó *et al*., 2011).

Numerosos organismos han mostrados efectividad contra fitopatógenos en una variedad de cultivos con alta importancia económica en condiciones controladas y en ensayos de campo a escala comercial (Nicot, 2011).Varios de estos microorganismos han sido formulados como productos comerciales. Sin embargo, actualmente hay muy pocos productos comercializados específicamente para el control de la PBC en viña y que muestren ser efectivos de forma consistente.

Tras 25 años de intenso trabajo de investigación y desarrollo de productos, y a pesar de todos estos aspectos positivos, su implementación a gran escala por los agricultores y la industria alimentaria/frutícola es en la actualidad muy reducida. Este hecho se ha subrayado ampliamente en diferentes publicaciones de los últimos años (Droby *et al*., 2009, Glare *et al*., 2012, Janisiewicz, 2010, Köhl *et al*., 2011, Montesinos, 2003, Nicot, 2011, Sharma *et al*., 2009, Spadaro y Gullino, 2004, Spadaro y Gullino, 2010, Teixidó *et al*., 2011). Los factores más importantes, según los citados documentos bibliográficos, que dificultan su adopción por el sector se pueden agrupar en cuatro bloques:

- **Aspectos regulatorios**, dado el continuo cambio de legislación entre regiones productivas y dentro de cada región. Por ejemplo, el caso de la UE y sus nuevas directivas No 1107/2009/EC que regulan la puesta en mercado de productos para la protección vegetal y No2009/128/EC que establece un marco de acción para la consecución de un uso racional de los pesticidas.

- **Aspectos técnicos.** La variabilidad en el control y los niveles de reducción menores a los de los pesticidas químicos hacen estos productos menos atractivos *a priori*. La causa de esta variabilidad suele residir en la falta de persistencia del control en el tiempo. Sin embargo, en muchos casos es dependiente de la falta de optimización de los tratamientos y de las condiciones en las que se realiza la aplicación, que no pueden ser siempre iguales que las de los productos químicos. Algunas líneas de investigación propuestas que pueden mejorar estos aspectos negativos son: el mejor conocimiento de sus modos de acción con las actuales técnicas moleculares, el enfoque multidisciplinar en la investigación, mayor énfasis en mejorar la supervivencia y la resistencia a estrés, optimización del momento y la cobertura de las aplicaciones, combinaciones de microorganismos con diferentes modos de acción (p.e. actividad en la filosfera y la rizosfera, competencia/inducción de resistencia).

- **Aspectos económicos**. Los productos basados en ACBs tienen, en general, un coste de producción más elevado que el de productos con moléculas químicas de síntesis y sobre todo hay pocas empresas capaces de hacerlo a gran escala. A este coste hay que sumar el coste de registro y el de distribución y comercialización. Estos factores hacen que el coste para el agricultor sea en ocasiones alto y que el margen de beneficios para las empresas que los comercializan sea reducido. Además, en la mayoría de los casos un ACB sólo es efectivo contra un patógeno y este reducido espectro de acción no es tampoco interesante para la industria de fitosanitarios.

- **Aspectos socioeconómicos**. El uso de estos productos por el sector agrícola depende mayoritariamente de la disponibilidad en el mercado, que está condicionada por la escasa voluntad de las empresas implicadas debido a algunos de los aspectos económicos ya comentados. Así mismo, la implementación de estos productos necesita de un importante trabajo por parte del sector público (gobiernos, universidades, agencias estatales) para: proporcionar información de las alternativas disponibles y beneficios, formar a los asesores, especialistas y agricultores sobre las características y modo de acción del producto para poder adaptar su uso y mejorar la efectividad, ofrecer incentivos y disponer de herramientas complementarias para mejorar su efectividad como Sistemas de Soporte de Decisiones. Otro aspecto que requiere el esfuerzo de toda las partes es asumir que el control biológico no puede alcanzar las tasas de reducción que presentan los químicos y que conseguir niveles de protección altos requiere un mayor esfuerzo de comprensión del agroecosistema, planificación y posiblemente combinación de estrategias

4.2. Control biológico de la PBC en viña

Distintos organismos han sido evaluados en el control de la PBC, incluyendo hongos filamentosos, bacterias y levaduras. Entre los hongos filamentosos, las diferentes especies del género *Trichoderma* han demostrado ser potencialmente efectivos y existen cepas antagonistas de hasta 6 especies. *T.harzianum*, *T. atroviride* y *T. viride* son las especies más efectivas y se encuentran formuladas en diferentes productos comerciales (Jacometti *et al.*, 2010). Su modo de acción es múltiple, e incluye estimulación del crecimiento y las defensas de las plantas, antibiosis, competencia colonización de tejidos necróticos (Elmer y Reglinski, 2006).

En Nueva Zelanda, el aislado HRU-3 del hongo *Ulocladium oudemansii* ha demostrado reducir significativamente la PBC en cosecha, mediante la supresión del patógeno en los tejidos necróticos del interior del racimo como modo de acción principal (Elmer *et al.*, 2007, Parry *et al.*, 2011, Reglinski *et al.*, 2005). Este organismo es el ingrediente activo del producto comercial Botry-Zen, registrado y en uso para agricultura ecológica en Nueva Zelanda. Su aplicación en campo se realiza en la primera parte de la campaña, en estrategias combinadas con productos naturales aplicados post-envero, consiguiendo programas de control de alta eficacia completamente independiente de fungicidas químicos (Reglinski *et al.*, 2010, Wurms *et al.*, 2011). Sin embargo, el mismo ACB no fue efectivo en otro estudio en viñedos cv. 'Neuburger' y 'Weisser Riesling' en Alemania, con condiciones muy diferentes (Mehofer *et al.*, 2009).

Muchas bacterias han demostrado ser antagonistas efectivos contra patógenos de diferentes cultivos, incluyendo los ACBs desarrollados en el centro IRTA-Lleida, *P. agglomerans* activo contra *Penicillium* spp. en fruta de pepita y en cítricos (Cañamás *et al.*, 2008a, Cañamás *et al.*, 2008c, Nunes *et al.*, 2001d) y *Bacillus subtilis* contra *Monilinia* spp. en fruta de hueso (Yánez–Mendizábal *et al.*, 2010). Los géneros a los que pertenecen buena parte de los antagonistas de *B. cinerea* son *Bacillus*, *Brevibacillus* y *Pseudomonas*. En viña, se cuenta con el producto Serenade® basado en *B. subtilis* y el producto Bio-Save® basado en *P. syringae*.

Levaduras y hongos tipo levadura son los microorganismos más frecuentemente utilizados en control biológico y su modo de acción generalmente es la competencia por nutrientes y espacio en los tejidos susceptibles de infección (Filonow, 1998).

Algunos organismos que han demostrado su efectividad contra *B. cinerea* en viña pertenecen al género *Candida*. Por ejemplo, *C. guillermondii* (Zahavi *et al.*, 2000), *C. oleophila* (El-Neshawy y El-Morsy, 2003), formulada en el producto Aspire® actualmente fuera del mercado, *C. saitoana* que se encuentra en los productos formulados BioCoat® y BioCure® (Elmer y Reglinski, 2006) y *C. sake* (Cañamás *et al.*, 2011), que trataremos en profundidad más adelante por ser el objeto de estudio de la presente tesis.

El género *Cryptococcus* también incluye varias especies antagonistas. En uva de mesa, *C. laurentii* redujo significativamente la incidencia de *B. cinerea* en conservación a 0 °C tras aplicaciones en pre-cosecha (Tian *et al.*, 2004) o post-cosecha combinada con quitosán (Meng y Tian, 2009). *C. humicolus* combinado con *P. anómala* y otros compuestos fue la combinación más efectiva tras frigoconservación y periodo de vida útil (Ligorio *et al.*, 2007).

Por último, también han mostrado efectividad organismos como *Metschnikowia pulcherrima* (Csutak *et al.*, 2013) y *M. fructicola* (Karabulut *et al.*, 2003, Kurtzman y Droby, 2001), así como *Saccharomyces chevalieri* (Sesan, 1999) y recientemente varias cepas de *S. cerevisiae* aisladas de la superficie de uvas en Argentina (Nally *et al.*, 2012), así como tres cepas de *Issatchenkia terrícola* en Chile (Vargas *et al.*, 2012).

Diferentes cepas del hongo tipo levadura *Aureobasidium pullulans*, han conseguido reducciones de la PBC en post-cosecha de uva de mesa (Lima *et al.*, 1997, Schena *et al.*, 2003). Parry *et al*. (2012) describieron el proceso de desarrollo de un hongo antagonista de este tipo, que mostró ser efectivo en campo como estrategia post-envero (Wurms *et al.*, 2011).

4.3. Candida sake *CPA-1*

Candida sake (Saito y Ota) (cepa CPA-1) es una levadura que fue aislada de la superficie de manzanas de frigoconservación por el grupo de Patología de la Post-cosecha del centro IRTA-Lleida y de la Universitat de Lleida en 1990. Posteriormente, esta cepa de *C. sake* fue depositada en la Colección Española de Cultivos Tipo (CECT-10817) en la Universidad de Valencia, situada en Burjassot.

Sus colonias son de color blanco cremoso, brillante, redondas con el borde liso y una ligera elevación central (Fig. 7a). La morfología de sus células es elíptica y alargada, como muestran los estudios con microscopía de barrido realizados, mientras que su forma de reproducción es vegetativa por gemación multilateral (Fig. 7b). Esta cepa es capaz de desarrollarse en un amplio rango de temperaturas, está muy bien adaptada a las bajas condiciones de frigoconservación (1 °C), pero también es capaz de multiplicarse a altas temperaturas (34 °C; Usall, 1995). Sin embargo, no presenta peligro toxicológico y su resistencia al contenido de etanol es limitada, por lo que implica un reducido riesgo de alterar la fermentación en el caso de la uva de vinificación.

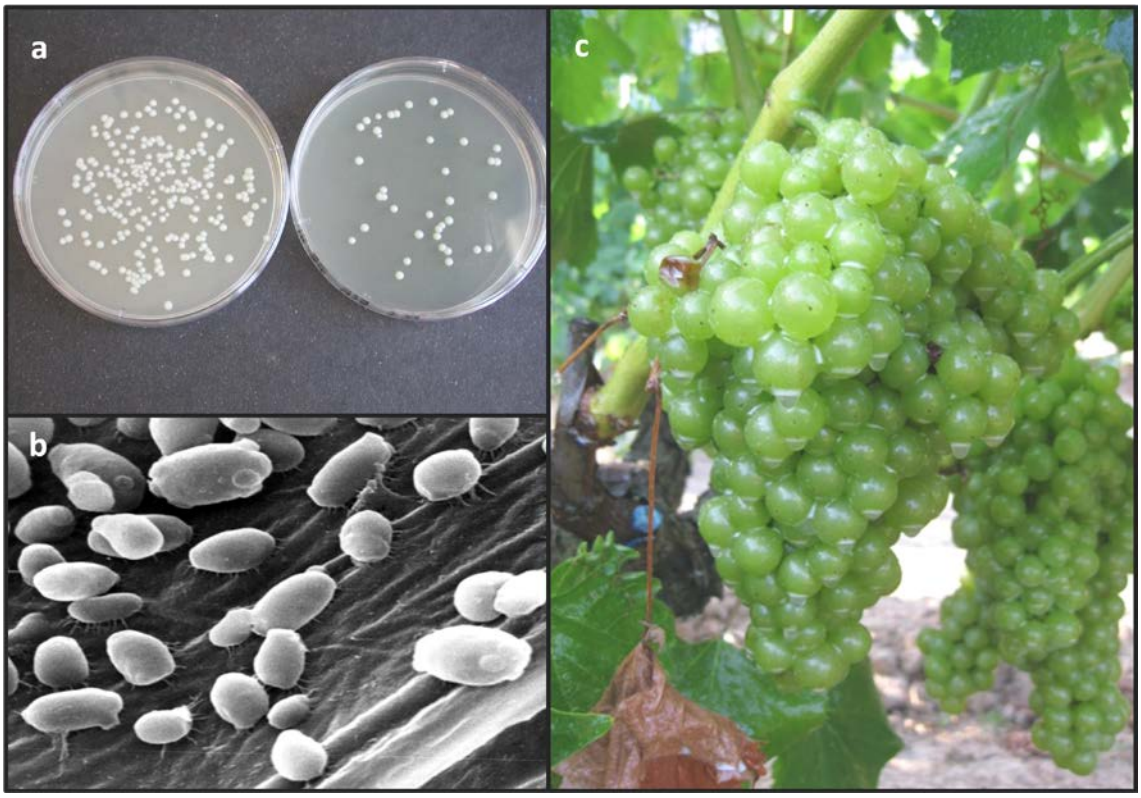


Figura 7 El agente de control biológico *Candida sake* CPA-1. a) morfología de las colonias de *C. sake* sobre medio NYDA, tras 48 horas de incubación a 22 C; b) Imagen de microscopio electrónico de barrido (SEM) de células de *C. sake* sobre la superficie de manzanas cv. Golden delicious, puede observarse su forma alargada y la presencia de gemas en su superficie, c) imagen de un racimo tras la aplicación en campo del tratamiento de *C. sake* y Fungicover.

Los estudios de efectividad con *C. sake* CPA-1 han mostrado su capacidad como antagonista en ensayos *in vitro* y sobre la superficie de peras y manzanas, mostrando reducciones muy altas de la infección de *Rhizopus* spp., *Penicillium expansum* y *B. cinerea*, alcanzando la supresión total del patógeno sobre heridas (Teixidó *et al.*, 1998b, Torres *et al.*, 2006, Usall, 1995, Viñas *et al.*, 1998). Además, su aplicación previa a cosecha consiguió también controlar *P. expansum* en manzanas durante el periodo post-cosecha y demostró su capacidad para sobrevivir a las condiciones de campo (Teixidó *et al.*, 1999). Su capacidad de supervivencia también ha sido mejorada por la completa caracterización ecofisiológica realizada por Teixidó *et al.* (1998a, d) y Usall (1995).

C. sake también fue efectiva en condiciones de aplicación semicomerciales y comerciales como ACB durante la cosecha (Usall *et al.*, 2000, 2001), mientras que su actividad antagonista fue incrementada mediante la adición de diferentes compuestos orgánicos (Nunes *et al.*, 2001a, 2001b, 2001c, 2002b), o por la aplicación conjunta con el ACB *P. agglomerans*, igualmente desarrollado en el centro de Lleida (Nunes *et al.*, 2002a).

Posteriormente se llevó a cabo la optimización del proceso de producción y formulación, durante los trabajos de la tesis doctoral de la Dra. M. Abadias (Abadias *et al.*, 2001a, Abadias *et al.*, 2001b, Abadias *et al.*, 2003a, Abadias *et al.*, 2005), que obtuvieron una formulación estable con alta viabilidad de las células del ACB (Abadias *et al.*, 2003b). Otros trabajos sobre la producción y formulación de *C. sake* se centraron en el desarrollo de reservas endógenas y el incremento de la resistencia al estrés ambiental de la cepa CPA-1 (Abadias *et al.*, 2000, Abadias *et al.*, 2001c, Cañamás *et al.*, 2008b, Teixidó *et al.*, 1998c, Torres *et al.*, 2003).

Finalmente, dada la efectividad mostrada por la levadura contra *B. cinerea* y su capacidad para tolerar situaciones de estrés en campo, varias de las cepas termotolerantes desarrolladas en estos estudios fueron evaluados para el control de *B. cinerea* en viña (Cañamás *et al.*, 2011). Los resultados mostraron alta eficacia y supervivencia de una da las cepas, sobre todo cuando fue aplicada en combinación con el aditivo Fungicover (BioDúrcal, Granada, España).

A la vista de la serie histórica de estudios realizados sobre esta cepa desde hace ya más de 20 años, *C. sake* CPA-1 ha superado actualmente la mayoría de los pasos necesarios antes de la comercialización y el uso a escala comercial de un ACB (Köhl *et al.*, 2011, Teixidó *et al.*, 2011), en este caso para la post-cosecha de fruta de pepita. El potencial del producto formulado despertó el interés de empresas del sector de fitosanitarios para su comercialización, llegando a presentarse a registro en España bajo la Orden APA/1470/2007 como producto fitofortificante para la prevención de enfermedades post-cosecha de pera y manzana por la empresa Sipcam-INAGRA (Valencia, España) con el nombre CANDIFRUIT®. Desgraciadamente, la empresa no ha llevado de forma exitosa esta última parte del proceso y el producto CANDIFRUIT fue introducido en el mercado durante una campaña pero en este momento no se comercializa ni puede ser aplicado de forma comercial en el sector de la post-cosecha de fruta. En la actualidad, la patente del producto formulado está de nuevo en posesión del IRTA.

En el caso de su aplicación en viña (Fig. 7c), varios aspectos relacionados con la optimización de los tratamientos en campo (dosis y momento de aplicación) estaban aún por determinar antes del presente trabajo, así como su posible integración en tratamientos combinados con otros productos. Su eficacia también debía ser confirmada con mayor número de campañas de ensayos con diferentes condiciones meteorológicas, varietales y de manejo del viñedo. Así mismo, la supervivencia bajos condiciones de campo sólo había sido evaluada en dos campañas y los factores que la determinaban se desconocían parcialmente. Por último, otra serie de aspectos más prácticos también necesitaban ser estudiados previamente a su aplicación comercial en viñedos, como la compatibilidad del ACB con aplicadores comerciales y con los productos fitosanitarios utilizados habitualmente por los viticultores.

La efectividad mostrada por el microorganismo en una variedad de situaciones y el avanzado estado de su desarrollo como producto representaban el mayor incentivo para continuar la investigación en la aplicación en viña de la cepa CPA-1 de *C. sake*. Por ello, las cuestiones referentes a la aplicación en campo enumeradas anteriormente forman el bloque central de los estudios desarrollados en la presente tesis doctoral.

4.4. Efectividad del control biológico y factores que la condicionan en campo

Uno de los mayores aspectos negativos que presentan los tratamientos con ACBs es la variabilidad en el control que presentan en algunas ocasiones, especialmente en la aplicación en campo. Esta variabilidad responde en gran medida a la falta de persistencia del microorganismo en altas concentraciones sobre la superficie de la planta, que lo hace inefectivo en momentos de gran vulnerabilidad a la infección.

Bajo condiciones de campo, el microorganismo antagonista se enfrenta a fluctuaciones de los factores bióticos y abióticos como T, HR, duración del periodo de rocío, disponibilidad de agua, manejo del cultivo, radiación solar, viento o lluvia, que pueden reducir drásticamente sus poblaciones (Köhl *et al.*, 2002, Magan, 2001)

Para maximizar la supervivencia del ACB tras su aplicación, el momento de aplicación es un factor muy importante, identificando momentos favorables para la colonización por parte del microorganismo, antes de momentos de alta susceptibilidad del huésped como la floración, el deshojado parcial o el periodo post-envero (Whipps y Lumsden, 2001). En el caso del control de la PBC, el momento de aplicación es especialmente importante, para que el antagonista se encuentre presente de forma preventiva antes de los momentos de fuerte desarrollo de la enfermedad (Elad y Stewart, 2004). Para conseguir un ajuste óptimo del momento de aplicación, por lo tanto, es necesario también un profundo conocimiento de la epidemiología del patógeno, del modo de acción del ACB y de su efecto sobre las diferentes vías de infección.

También es importante la selección de un antagonista que esté adaptado constitutivamente a las condiciones de estrés a las que se verá sometido en campo (Köhl *et al.*, 2002), o utilizar antagonistas adaptados a estrés durante el proceso de producción y formulación (Cañamás *et al.*, 2008b, Cañamás *et al.*, 2011).

Así mismo, la estrategia que se sugiere como más efectiva a nivel práctico para reducir la variabilidad en el control es la combinación de mecanismos de acción, mediante la aplicación conjunta de varios microorganismos (Guetsky *et al.*, 2002a, Guetsky *et al.*, 2002b), la combinación con químicos (Elad y Stewart, 2004), o sobre todo la combinación con productos naturales que puedan favorecer la supervivencia y eficacia del ACB, dando lugar a una formulación de aplicación (Cañamás *et al.*, 2008c, Cañamás *et al.*, 2011, Lahlali *et al.*, 2011, Lima *et al.*, 2005, Qin *et al.*, 2006, Teixidó *et al.*, 2010, Tesfagiorgis y Annegarn, 2013).

5. Productos naturales

5.1. ¿Qué conocemos como productos naturales?

Los productos naturales se definen de forma general como ingredientes activos de origen vegetal, animal o microbiano capaces de reducir el desarrollo de un patógeno a través de la actividad antifúngica directa, formación de biofilms o estimulación de las defensas de la planta (Romanazzi *et al.*, 2012, Tripathi y Dubey, 2004). Se trata de sustancias que *a priori* tienen reducido impacto medioambiental y baja toxicidad para organismos que no son su objetivo de control.

Muchas de estas sustancias, por lo tanto son categorizadas como GRAS ("Generally Recognized As Safe"), una denominación acuñada por la US Food and Drug Administration (Nicot, 2011). En esta sección, añadiremos también los compuestos inorgánicos dado su origen no sintético.

5.2. Compuestos inorgánicos

Entre los métodos alternativos a los fungicidas químicos, la aplicación de sales minerales representa una alternativa estudiada y con mucho potencial, dada su fácil disponibilidad y bajo coste. Sus aplicaciones para el control de hongos fitopatogénicos han sido recientemente revisadas por Deliopoulos *et al.* (2010).

El calcio inhibe el desarrollo de *B. cinerea* (Chardonnet *et al*., 2000), mientras que incrementa los niveles de calcio en los tejidos de la viña, haciéndolos más resistentes a la infección (Elmer y Reglinski, 2006). Nigro *et al.* (2006) evaluaron hasta 19 sales, de las cuales el cloruro cálcico, bicarbonato sódico y carbonato sódico lograron entre el 37-53% de reducción de la severidad de *B. cinerea* en post-cosecha de uva de mesa con aplicaciones pre-cosecha. Recientemente, la efectividad de ocho sales de calcio y potasio ha sido también demostrada, consiguiendo reducciones del 77-100% de la incidencia comparado con el control tras conservación frigorífica y periodo de vida útil (Youssef y Roberto, 2014). En estudios recientes, Feliziani *et al*. (2013) también han observado reducciones de la PBC en post-cosecha con aplicaciones de sorbato potásico. Además el potencial de estas sales inorgánicas es mayor, ya que también pueden ser utilizadas en combinación con ACBs (An *et al*., 2013, Ippolito *et al*., 2005, Ligorio *et al*., 2007, Teixidó *et al*., 2001).

5.3. Preparados de compost y extractos vegetales

La aplicación de preparados de compost y preparados vegetales es una práctica habitual en horticultura y fruticultura ecológicas, que también es utilizada en viña, aunque su efectividad está pobremente documentada en publicaciones científicas.

Los preparados de compost consisten en la fermentación aeróbica de compost diluido en agua que es aplicado vía foliar, aportando un preparado nutritivo y con una fuerte carga microbiológica activada, que estimulan el crecimiento y los mecanismos de resistencia de la planta huésped. Su efectividad es muy dependiente de la calidad del compost utilizado y de su composición, mientras que la población microbiana contenida representa una parte importante de su eficacia (Elmer y Reglinski, 2006, Suarez-Estrella *et al*., 2013). Su efectividad contra *B. cinerea* está descrita en diferentes patosistemas (El-Masry *et al*., 2002, Palmer *et al*., 2010, Segarra *et al*., 2007, Termorshuizen *et al*., 2006), y diversos estudios han evaluado su aplicación en viña contra la PBC (Elad y Shtienberg, 1994, Elmer y Reglinski, 2006) y más recientemente Evans *et al.* (2013). Aunque las reducciones obtenidas de la PBC no son muy elevadas, esta estrategia tiene la ventaja de actuar como fertilizante y fortalecer la planta contra otras importantes enfermedades como el oídio de la vid, causado por *Erysiphe necator* (syn: *Uncinula necator*) (Elad y Shtienberg, 1994, Evans *et al.*, 2013).

En cuanto a los extractos vegetales, algunos pueden encontrarse como productos comercializados. Es el caso de Milsana®, basado en *Reynoutria sachalinensis*, capaz de reducir la PBC con aplicaciones de campo durante toda la campaña en ensayos realizados en Alemania (Mehofer *et al.*, 2009, Schilder *et al.*, 2002). Recientemente varios extractos de plantas silvestres comestibles han sido evaluados contra los principales patógenos de post-cosecha de fruta, encontrando que *Sanguisorba minor* inhibió notablemente la germinación de *B. cinerea* y controló su infección sobre bayas heridas (Gatto *et al.*, 2011). Los extractos (hexano y cloroformo) de los residuos de bodega o vinazas, mostraron también inhibición del crecimiento de *B. cinerea in vitro*, representando una nueva fuente de posibles sustancias con un reducido coste (Mendoza *et al.*, 2013).

Como extractos vegetales deben incluirse también los extractos de algas. Jeandet *et al.* (1996) observaron reducciones efectivas de la PBC usando el extracto de algas Synermix® en una serie de 8 años de estudios en Francia, aplicando una estrategia combinada con iprodiona. El polisacárido extraído de algas Ulvan también inhibió a *B. cinerea*en experimentos *in vitro*, pero no fue efectivo en aplicaciones sobre uva en campo (Montealegre *et al.*, 2010). El extracto del alga marrón *Lessonia trabeculata* también ha mostrado su efectividad contra *B. cinerea* en tomate (Jimenez *et al.*, 2011).

Los aceites esenciales son otro método ampliamente estudiado en la post-cosecha de fruta. En el caso de uva de mesa, se han publicado reducciones significativas aplicando aceite de hoja de canela (Melgarejo-Flores *et al.*, 2013), aceites de orégano y limón (Vitoratos *et al.*, 2013) y tomillo (Valero *et al.*, 2006, Walter *et al.*, 2001), aunque este último produjo fitotoxicidad en las flores.

También mencionaremos aquí el uso de las hormonas vegetales giberelinas, utilizadas para promover el desarrollo de racimos más laxos y menos compactos, que han demostrado también reducir eficazmente la PBC en campo (Evers *et al.*, 2010, Mehofer, 2011, Spring y Viret, 2011). El mismo efecto se ha documentado para el regulador vegetal prohexadiona-calcio (Schildberger *et al.*, 2011).

5.4. Inductores de las defensas de las plantas

Varias sustancias tanto orgánicas como inorgánicas han mostrado activar mecanismos de defensa de las plantas. Elmer y Reglinski (2006) revisaron las sustancias que han mostrado este efecto sobre la viña, destacando entre ellas el ácido salicílico, el ácido β-Aminobutírico, el calcio y el ácido jasmónico. Estas sustancias han mostrado una eficacia moderada y efectos fitotóxicos en algunos casos.

Otro producto que ha ofrecido resultados prometedores es el quitosán, un polímero derivado de la quitina, cuyo modo de acción reside en la activación de defensas de la planta pero también muestra actividad antifúngica directa. Por sus características de baja toxicidad, es ampliamente utilizado en la prevención de patologías post-cosecha de fruta, como ha sido revisado por Zhang *et al.* (2011). En viña se ha aplicado contra la PBC en dosis entre 0.5 % (v/v) y 2% (v/v), en aplicaciones post-cosecha sobre uva de mesa (Meng *et al.*, 2008, Romanazzi *et al.*,

2009, Romanazzi *et al.*, 2012, Xu *et al.*, 2007), mientras que otros estudios han evaluado aplicaciones pre-cosecha en campo en los últimos años, mostrando buenos resultados (Feliziani *et al.*, 2013, Meng y Tian, 2009, Reglinski *et al.*, 2010, Romanazzi *et al.*, 2002, Romanazzi *et al.*, 2006, Wurms *et al.*, 2011), pero inefectivo en otros casos (Montealegre *et al.*, 2010).

5.5. *Productos naturales basados en ácidos grasos*

La aplicación de formulaciones basadas en ácidos grasos ha demostrado su efectividad en el control de numerosos hongos fitopatogénicos incluidos por ejemplo el mildiu y el oídio de la viña (Palla, 2006, Vercesi *et al.*, 2001). En el caso de *B. cinerea* , un derivado del ácido esteárico mostró ser efectivo en el control de *Botrytis* spp. en ensayos *in vitro* (Hou y Forman, 2000), mientras que Montealegre *et al.* (2010) observaron control efectivo de *B. cinerea* en post-cosecha de manzanas usando una formulación comercial de ácidos grasos y otras sustancias.

El producto comercial Fungicover® Base (Biodúrcal S.L., Granada, España) es una emulsión de ácidos grasos en medio acuoso-alcohólico, cuyo uso está recomendado para la prevención de varias enfermedades fúngicas, incluida la PBC de la viña y está actualmente certificado para su uso en agricultura ecológica en España (Fig 8a).

Protector^{HML} (PRT; Henry Manufacturing Limited, Nueva Zelanda) es un producto basado en sales potásicas de ácidos grasos, registrado para uso en viticultura convencional y ecológica para el tratamiento del oídio y la PBC en viña (Fig 8b). Los resultados de su aplicación en sucesivas campañas vitícolas en Nueva Zelanda pueden consultarse en la página web del fabricante (http://www.henrymanufacturing.co.nz/products/protectorhml/).

Figure 8 Productos comerciales basados en ácidos grasos para su aplicación en viña contra la podredumbre por *B. cinerea*. a) envase del producto Fungicover Base, producido en Granada por la empresa BioDurcal S.A; b) envase del producto Protector, registrado y comercializado en Nueva Zelanda por la empresa Henry Manufacturing Limited.

También en Nueva Zelanda, se encuentra en las últimas fases de su desarrollo el producto NP2, basado en componentes grasos de la leche de vaca y que ha demostrado su eficacia al ser aplicado en el cierre de racimo, dentro de programas integrados con otros métodos no químicos de control de la PBC (Wurms *et al*., 2011).

Una de las mayores ventajas que presentan estos productos basados en ácidos grasos es su bajo coste de fabricación, dada su abundancia en la naturaleza y su facilidad de extracción (Ruiz-Rodriguez *et al*. 2010). Además, sus propiedades adherentes y capacidad de formar películas sobre la superficie del huésped, los convierte en buenos candidatos para aplicar como aditivos de ACBs. Este es el caso de la aplicación de Protector^{HML} con *U. oudemansii*, o de la aplicación de Fungicover que se ha comprobado en estudios precedentes que mejora la supervivencia en campo de *C. sake* (Cañamás *et al*., 2008a) y otros agentes de biocontrol (Cañamás *et al*., 2008b).

REFERENCIAS

Abadias M, Teixidó N, Usall J, Viñas I y Magan N, 2000. Solute stresses affect growth patterns, endogenous water potentials and accumulation of sugars and sugar alcohols in cells of the biocontrol yeast *Candida sake*. *Journal of Applied Microbiology*, **89**, 1009-1017.

Abadias M, Benabarre A, Teixidó N, Usall J y Viñas I, 2001a. Effect of freeze drying and protectants on viability of the biocontrol yeast *Candida sake*. *International Journal of Food Microbiology*, **65**, 173-182.

Abadias M, Teixidó N, Usall J, Benabarre A y Viñas I, 2001b. Viability, efficacy, and storage stability of freeze-dried biocontrol agent *Candida sake* using different protective and rehydration media. *Journal of Food Protection*, **64**, 856-861.

Abadias M, Teixidó N, Usall J, Viñas I y Magan N, 2001c. Improving water stress tolerance of the biocontrol yeast *Candida sake* grown in molasses-based media by physiological manipulation. *Canadian Journal of Microbiology*, **47**, 123-129.

Abadias M, Teixidó N, Usall J y Viñas I, 2003a. Optimization of growth conditions of the postharvest biocontrol agent *Candida sake* CPA in a lab-scale fermenter. *Journal of Applied Microbiology*, **95**, 301-309.

Abadias M, Usall J, Teixidó N y Viñas I, 2003b. Liquid formulation of the postharvest biocontrol agent *Candida sake* CPA-1 in isotonic solutions. *Phytopathology*, **93**, 436-442.

Abadias M, Teixidó N, Usall J, Solsona C y Viñas I, 2005. Survival of the postharvest biocontrol yeast *Candida sake* CPA-1 after dehydration by spray-drying. *Biocontrol Science and Technology*, **15**, 835-846.

Agnew RH, Mundy DC y Balasubramaniam R, 2004 Effects of spraying strategies based on monitored disease risk on grape disease control and fungicide usage in Marlborough. In: Zydenbos S. M., ed. *Proceedings of the 57th Annual Meeting of the New-Zealand-Plant-Protection-Society, 2004. Hamilton, NEW ZEALAND.* 30-36.

Agrios GN, 1995. *Fitopatologia / Plant Pathology*. New York: Academic Press.

Aira MJ, Fernandez-Gonzalez M, Rodriguez-Rajo FJ y Jato V, 2009. Predictive model for *Botrytis cinerea* in a vineyard of Galicia, Spain. *Boletín Micológico*, **24**, 27-35.

Alonso-Villaverde V, Boso S, Santiago JL, Gago P y Martinez MC, 2008. Relationship between susceptibility to *Botrytis* bunch rot and grape cluster morphology in the *Vitis vinifera* L. cultivar Albarino. *International Journal of Fruit Science*, **8**, 251-265.

An B, Li B, Qin G y Tian S, 2013. Exogenous calcium improves viability of biocontrol yeasts under heat stress by reducing ROS accumulation and oxidative damage of cellular protein. *Current Microbiology*, **65**, 122-127.

Barata A, Malfeito-Ferreira M y Loureiro V, 2012a. The microbial ecology of wine grape berries. *International Journal of Food Microbiology*, **153**, 243-259.

Barata A, Santos SC, Malfeito-Ferreira M y Loureiro V, 2012b. New insights into the ecological interaction between grape berry microorganisms and *Drosophila* flies during the development of sour rot. *Microbial Ecology*, **64**, 416-430.

Beever RE y Weeds PL, 2004. Taxonomy and genetic variation of *Botrytis* and *Botryotinia*. En: Elad Y., Williamson B., Tudzynski P. and Delen N., eds. *Botrytis: biology, pathology and control.* 29-52.

Bretveld R, Brouwers M, Ebisch I y Roeleveld N, 2007. Influence of pesticides on male fertility. *Scandinavian Journal of Work, Environment and Health*, **33**, 13-28.

Broome JC, English JT, Marois JJ, Latorre BA y Aviles JC, 1995. Development of an infection model for *Botrytis* bunch rot of grapes based on wetness duration and temperature. *Phytopathology*, **85**, 97-102.

Cañamás TP, Viñas I, Usall J, Casals C, Solsona C y Teixidó N, 2008a. Control of postharvest diseases on citrus fruit by preharvest application of the biocontrol agent *Pantoea agglomerans* CPA-2 part I. Study of different formulation strategies to improve survival of cells in unfavourable environmental conditions. *Postharvest Biology and Technology*, **49**, 86-95.

Cañamás TP, Viñas I, Usall J, Magan N, Solsona C y Teixidó N, 2008b. Impact of mild heat treatments on induction of thermotolerance in the biocontrol yeast *Candida sake* CPA-1 and viability after spray-drying. *Journal of Applied Microbiology*, **104**, 767-775.

Cañamás TP, Viñas I, Usall J, Torres R, Anguera M y Teixidó N, 2008c. Control of postharvest diseases on citrus fruit by preharvest applications of biocontrol agent *Pantoea agglomerans* CPA-2 part II. Effectiveness of different cell formulations. *Postharvest Biology and Technology*, **49**, 96-106.

Cañamás TP, Viñas I, Torres R, Usall J, Solsona C y Teixidó N, 2011. Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. *Biological Control*, **56**, 150-158.

Chardonnet CO, Sams CE, Trigiano RN y Conway WS, 2000. Variability of three isolates of *Botrytis cinerea* affects the inhibitory effects of calcium on this fungus. *Postharvest Pathology and Mycotoxins*, **90**, 769-774.

Chebil S, Roudet J, Dubos B y Ghorbel A, 2003. Table grapes in Tunisia. Optimization of protection with regard to grey mould. *Arboriculture Fruitiere*, **574**, i-v.

Coertze S, Holz G y Sadie A, 2001. Germination and establishment of infection on grape berries by single airborne conidia of *Botrytis cinerea*. *Plant Disease*, **85**, 668-677.

Coertze S y Holz G, 2002. Epidemiology of *Botrytis cinerea* on grape: wound infection by dry, airborne conidia. *South African Journal for Enology and Viticulture*, **23**, 72-77.

Crane M, Norton A, Leaman J, Chalak A, Bailey A, Yoxon M, Smith J y Fenlon J, 2006. Acceptability of pesticide impacts on the environment: what do United Kingdom stakeholders and the public value? *Pest Management Science*, **62**, 5-19.

Cravero S, Valle Dd y Rabino M, 2004. Manual and mechanical grapevine leaf removal: effects on grey mould, sour rot and other grape diseases, comparison with chemical control. *Giornate Fitopatologische*, 227-234.

Csutak O, Vassu T, Sarbu I, Stoica I y Cornea P, 2013. Antagonistic activity of three newly isolated yeast strains from the surface of fruits. *Food Technology and Biotechnology*, **51**, 70-77.

Cus F, Cesnik HB, Bolta SV y Gregorcic A, 2010. Pesticide residues and microbiological quality of bottled wines. *Food Control*, **21**, 150-154.

Darriet P, Bouchilloux P, Poupot C, Bugaret Y, Clerjeau M, Sauris P, Medina B y Dubourdieu D, 2001. Effects of copper fungicide spraying on volatile thiols of the varietal aroma of Sauvignon blanc, Cabernet Sauvignon and Merlot wines. *Vitis*, **40**, 93-99.

Deliopoulos T, Kettlewell PS y Hare MC, 2010. Fungal disease suppression by inorganic salts: A review. *Crop Protection*, **29**, 1059-1075.

Deytieux-Belleau C, Geny L, Roudet J, Mayet V, Doneche B y Fermaud M, 2009. Grape berry skin features related to ontogenic resistance to *Botrytis cinerea*. *European Journal of Plant Pathology*, **125**, 551-563.

Diez-Navajas AM y Ortiz-Barredo A, 2011. Downy, powdery mildew and botrytis risk assessment by climate parameters, pest and spores monitoring in Rioja Alavesa vineyard (Northwest of Spain). *IOBC/WPRS Bulletin*, **67**, 21-29.

Diez A, 2010. Enfermedades fúngicas foliares de la vid: mildiu, oidio y botritis. En: Neiker-Tecnalia, ed. *Publicaciones divulgativas - Producción y protección vegetal.* Online (http://www.neiker.net/datos/documentos/2010224Enfermedades_fungicas_foliares.pdf ; Acceso 31/10/2013)

Droby S, Wisniewski M, Macarisin D y Wilson C, 2009. Twenty years of postharvest biocontrol research: Is it time for a new paradigm? *Postharvest Biology and Technology*, **52**, 137-145.

El-Masry MH, Khalil AI, Hassouna MS y Ibrahim HAH, 2002. In situ and in vitro suppressive effect of agricultural composts and their water extracts on some phytopathogenic fungi. *World Journal of Microbiology & Biotechnology*, **18**, 551-558.

El-Neshawy SM y El-Morsy FM, 2003. Control of gray Mold of grape by pre-harvest application of Candida oleophila and its combination with a low dosage of Euparen. *Proceedings of the 8th International Controlled Atmosphere Research Conference, Vols I and Ii*, 95-102.

Elad Y y Shtienberg D, 1994. Effect of compost water extracts on grey mould (*Botrytis cinerea*). *Crop Protection*, **13**, 109-114.

Elad Y y Stewart A, 2004. Microbial control of *Botrytis* spp. En: Elad Y., Williamson B., Tudzynski P. and Delen N., eds. *Botrytis: biology, pathology and control.* Dordrecht, the Netherlands: Kuwer academic publishers, 223-241.

Elad Y, Williamson B, Tudzynski P y Delen N, eds, 2004a. *Botrytis: Biology, Pathology and control*. Dordrecht, the Netherlands: Kuwer academic publishers.

Elad Y, Williamson B, Tudzynski P y Delen N, 2004b. *Botrytis* spp. and diseases they cause in agricultural systems - an introduction. En: Elad Y., Williamson B., Tudzynski P. and Delen N., eds. *Botrytis: biology, pathology and control.* 1-8.

Elmer PAG y Michailides TJ, 2004. Epidemiology of *Botrytis cinerea* in orchard and vine crops. En: Elad Y., Williamson B., Tudzynski P. and Delen N., eds. *Botrytis: Biology, Pathology and control.* Dordrecht, the Netherlands: Kuwer academic publishers, 243-272.

Elmer PAG y Reglinski T, 2006. Biosuppression of *Botrytis cinerea* in grapes. *Plant Pathology*, **55**, 155-177.

Elmer PAG, Reglinski T, Wurms K y Wood PN, 2007. Enabling technologies: the practical integration of biological and natural products to disease control. En: Butcher M. R., Walker J. T. S. and Zydenbos S. M., eds. *Proceedings of the Future changes in crop protection: Repositioning NZ's Primary Industries*. *Napier, New Zealand.* 109-116.

Engelbrecht R, Holz G y Pringle KL, 2004. Occurrence of fruit-decaying fungi on adult male mediterranean fruit flies (*Ceratitis capitata*) captured in orchards and adjacent vineyards. *South African Journal of Enology and Viticulture*, **25**, 48-53.

Evans KJ, Palmer AK y Metcalf DA, 2013. Effect of aerated compost tea on grapevine powdery mildew, botrytis bunch rot and microbial abundance on leaves. *European Journal of Plant Pathology*, **135**, 661-673.

Evers D, Molitor D, Rothmeier M, Behr M, Fischer S y Hoffmann L, 2010. Efficiency of different strategies for the control of grey mold on grapes including gibberellic acid (Gibb3), leaf

removal and/or botrycide treatments. *Journal International des Sciences de la Vigne et du Vin*, **44**, 151-159.

Farquhar SA, Goff NM, Shadbeh N, Samples J, Ventura S, Sanchez V, Rao P y Davis S, 2009. Occupational health and safety status of indigenous and Latino farmworkers in Oregon. *Journal of Agricultural Safety and Health*, **15**, 89-102.

Feliziani E, Smilanick JL, Margosan DA, Mansour MF, Romanazzi G, Gu S, Gohil HL y Rubio Ames Z, 2013. Preharvest fungicide, potassium sorbate, or chitosan use on quality and storage decay of table grapes. *Plant Disease*, **97**, 307-314.

Fermaud M y Lemenn R, 1989. Association of *Botrytis cinerea* with grape berry moth larvae. *Phytopathology*, **79**, 651-656.

Fermaud M y Giboulot A, 1992. Influence of *Lobesia botrana* larvae on field severity of *Botrytis* rot of grape berries. *Plant Disease*, **76**, 404-409.

Fermaud M y Lemenn R, 1992. Transmission of *Botrytis cinerea* to grapes by grape berry moth larvae. *Phytopathology*, **82**, 1393-1398.

Fermaud M y Gaunt RE, 1995. *Thrips obscuratus* as a potential vector of *Botrytis cinerea* in kiwifruit *Mycological Research*, **99**, 267-273.

Fermaud M, 1998. Cultivar susceptibility of grape berry clusters to larvae of *Lobesia botrana* (Lepidoptera : Tortricidae). *Journal of Economic Entomology*, **91**, 974-980.

Fermaud M, Gravot E, Blancard D, Jailloux F y Stockel J, 2000. Association between *Drosophila* and micro-organisms in vineyards in Bordeaux damaged by sour rot. *Bulletin OILB/SROP*, **23**, 55-57.

Fermaud M, Martinez F, Pieri P, Froidefond G, Sauris P y Delière L, 2003. Pourriture grise de la vigne. Prévision du risque épidémique et protection fongicide. *Phytoma*, **565**, 35-40.

Fermaud M, Roudet J, Sauris P, Druelle L, Blanc G y Lagouarde P, 2010. Implementation of a low-input fungicide strategy in relation to the pattern of grey mold development. *Proceedings of the XV International* Botrytis *Symposium*. *Cadiz, Spain.*

Filonow AB, 1998. Role of competition for sugars by yeasts in the biocontrol of gray mold of apple. *Biocontrol Science and Technology*, **8**, 243-256.

Fourie JF y Holz G, 1994. Infection of plum and nectarine flowers by *Botrytis cinerea*. *Plant Pathology*, **43**, 309-315.

Fourie JF y Holz G, 1995. Initial infection processes by *Botrytis cinerea* on nectarine and plum fruit and the development of decay. *Phytopathology*, **85**, 82-87.

Galet P, 1999. *Précis de pathologie viticole*. Montpellier: Lavoisier.

Gatto MA, Ippolito A, Linsalata V, Cascarano NA, Nigro F, Vanadia S y Di Venere D, 2011. Activity of extracts from wild edible herbs against postharvest fungal diseases of fruit and vegetables. *Postharvest Biology and Technology*, **61**, 72-82.

Gil E, 2003. *Tratamientos en viña : equipos y técnicas de aplicación*. Barcelona: Edicions UPC.

Gil E, Llorens J, Landers A, Llop J y Giralt L, 2011. Field validation of DOSAVIÑA, a decision support system to determine the optimal volume rate for pesticide application in vineyards. *European Journal of Agronomy*, **35**, 33-46.

Glare T, Caradus J, Gelernter W, Jackson T, Keyhani N, Köhl J, Marrone P, Morin L y Stewart A, 2012. Have biopesticides come of age? *Trends in Biotechnology*, **30**, 250-258.

Goetz G, Fkyerat A, Metais N, Kunz M, Tabacchi R, Pezet R y Pont V, 1999. Resistance factors to grey mould in grape berries: identification of some phenolics inhibitors of *Botrytis cinerea* stilbene oxidase. *Phytochemistry*, **52**, 759-767.

Gonzalez-Alvarez M, Gonzalez-Barreiro C, Cancho-Grande B y Simal-Gandara J, 2012. Impact of phytosanitary treatments with fungicides (cyazofamid, famoxadone, mandipropamid and valifenalate) on aroma compounds of Godello white wines. *Food Chemistry*, **131**, 826-836.

Gravot E, Blancard D, Fermaud M, Lonvaud A y Joyeux A, 2001. Sour rot. I: Etiology. Research into the causes of this form of rot of grapes in Bordeaux vineyards. *Phytoma*, 36-39.

Grosman J, Micoud A, Remuson F, Guellec Ml y Panon ML, 2012. Grey-mould resistance to fungicides on grapevine-results of the monitoring directed by the French plant protection organization and the Champagne Committee between 2008 and 2011. *10e Conference Internationale sur les Maladies des Plantes, Tours, France, 3, 4 & 5 Decembre.* 249-259.

Guerzoni E y Marchetti R, 1987. Analysis of yeast flora associated with grape sour rot and of the chemical disease markers. *Applied and Environmental Microbiology*, **53**, 571-576.

Guetsky R, Elad Y, Shtienberg D y Dinoor A, 2002a. Establishment, survival and activity of the biocontrol agents *Pichia guilliermondii* and *Bacillus mycoides* applied as a mixture on strawberry plants. *Biocontrol Science and Technology*, **12**, 705-714.

Guetsky R, Shtienberg D, Elad Y, Fischer E y Dinoor A, 2002b. Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology*, **92**, 976-985.

Hanni E, Lardschneider E y Kelderer M, 2013. Alternatives to the use of gibberellins for bunch thinning and bunch compactness reduction on grapevine. *Acta Horticulturae.* **978**, 335-345.

Hed B, Ngugi HK y Travis JW, 2009. Relationship between cluster compactness and bunch rot in Vignoles grapes. *Plant Disease*, **93**, 1195-1201.

Holz G, Coertze S y Williamson B, 2004. The ecology of *Botrytis* on plant surfaces. En: Elad Y., Williamson B., Tudzynski P. and Delen N., eds. *Botrytis: biology, pathology and control.* Dordretch, The Netherlands: Kuwer academic publishers, 9-27.

Hou CT y Forman RJ, 2000. Growth inhibition of plant pathogenic fungi by hydroxy fatty acids. *Journal of Industrial Microbiology & Biotechnology*, **24**, 275-276.

Huber C, McFadden-Smith W y Inglis D, 2011. Management and etiology of grape sour rot in the Niagara region. *Phytopathology*, **101**, S259-S259.

Ippolito A, Schena L, Pentimone I y Nigro F, 2005. Control of postharvest rots of sweet cherries by pre- and postharvest applications of *Aureobasidium pullulans* in combination with calcium chloride or sodium bicarbonate. *Postharvest Biology and Technology*, **36**, 245-252.

Jacometti MA, Wratten SD y Walter M, 2007. Management of understorey to reduce the primary inoculum of *Botrytis cinerea*: Enhancing ecosystem services in vineyards. *Biological Control*, **40**, 57-64.

Jacometti MA, Wratten SD y Walter M, 2010. Review: Alternatives to synthetic fungicides for *Botrytis cinerea* management in vineyards. *Australian Journal of Grape and Wine Research*, **16**, 154-172.

Janisiewicz WJ, 2010. Quo Vadis of Biological Control of Postharvest Diseases. En: Prusky D. and Gullino M. L., eds. *Postharvest Pathology.* (Plant Pathology in the 21st Century; vol. 2). Springer Netherlands, 137-148.

Jarvis W, 1980. Epidemiology. En: Coley-Smith J., Verhoef K. and Jarvis W., eds. *The biology of Botrytis.* London, UK: Academic press, 219-250.

Jaspers MV, Seyb AM, Trought MCT y Balasubramaniam R, 2013. Overwintering grapevine debris as an important source of *Botrytis cinerea* inoculum. *Plant Pathology*, **62**, 130-138.

Jeandet P, Adrian M, Joubert JM, Hubert F y Bessis R, 1996. Stimulating the natural defences of grape. A complement to phytosanitary control of *Botrytis*. *Phytoma*, 21-22, 24-25.

Jersch S, Scherer C, Huth G y Schlosser E, 1989. Proanthocyanidins as basis for quiescence of *Botrytis cinerea* in immature strawberry fruits. *Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection*, **96**, 365-378.

Jimenez E, Dorta F, Medina C, Ramirez A, Ramirez I y Pena-Cortes H, 2011. Anti-Phytopathogenic Activities of Macro-Algae Extracts. *Marine Drugs*, **9**, 739-756.

Karabulut OA, Smilanick JL, Gabler FM, Mansour M y Droby S, 2003. Near-harvest applications of *Metschnikowia fructicola*, ethanol, and sodium bicarbonate to control postharvest diseases of grape in central California. *Plant Disease*, **87**, 1384-1389.

Keller M, Kummer M y Vasconcelos MC, 2001. Reproductive growth of grapevines in response to nitrogen supply and rootstock. *Australian Journal of Grape and Wine Research*, **7**, 12-18.

Keller M, Viret O y Cole FM, 2003. *Botrytis cinerea* infection in grape flowers: Defense reaction, latency, and disease expression. *Phytopathology*, **93**, 316-322.

Köhl J, Molhoek WML, Lombaers-van der Plas C, Goossen-van de Geijn H, de Haas L y Gerlagh T, 2002. Effect of environmental factors on conidial germination of the *Botrytis* spp. antagonist *Ulocladium atrum*. *Bulletin OILB/SROP*, **25**, 65-68.

Köhl J, Postma J, Nicot P, Ruocco M y Blum B, 2011. Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. *Biological Control*, **57**, 1-12.

Komárek M, Čadková E, Chrastný V, Bordas F y Bollinger JC, 2010. Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. *Environment International*, **36**, 138-151.

Kurtzman CP y Droby S, 2001. *Metschnikowia fructicola*, a new ascosporic yeast with potencial for biocontrol of postharvest fruit rot. *Systematic and Applied microbiology*, **24**, 395-399.

Ky I, Lorrain B, Jourdes M, Pasquier G, Fermaud M, Geny L, Rey P, Doneche B y Teissedre PL, 2012. Assessment of grey mould (*Botrytis cinerea*) impact on phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages. *Australian Journal of Grape and Wine Research*, **18**, 215-226.

Lahlali R, Raffaele B y Jijakli MH, 2011. UV protectants for *Candida oleophila* (strain O), a biocontrol agent of postharvest fruit diseases. *Plant Pathology*, **60**, 288-295.

Latorre BA, Rioja ME y Lillo C, 2002a. Effect of temperature on flower and berry infections caused by *Botrytis cinerea* on table grapes. *Ciencia e Investigación Agraria*, **29**, 145-151.

Latorre BA, Spadaro I y Rioja ME, 2002b. Occurrence of resistant strains of *Botrytis cinerea* to anilinopyrimidine fungicides in table grapes in Chile. *Crop Protection*, **21**, 957-961.

Latorre BA y Torres R, 2012. Prevalence of isolates of *Botrytis cinerea* resistant to multiple fungicides in Chilean vineyards. *Crop Protection*, **40**, 49-52.

Leroch M, Kretschmer M y Hahn M, 2011. Fungicide resistance phenotypes of *Botrytis cinerea* isolates from commercial vineyards in south west Germany. *Journal of Phytopathology*, **159**, 63-65.

Leroux P, 2004. Chemical control of *Botrytis* and its resistance to chemical fungicides. En: Elad Y., Williamson B., Tudzynski P. and Delen N., eds. *Botrytis: biology, pathology and control.* Dordrecht, the Netherlands: Kuwer academic publishers, 195-222.

Ligorio A, Platania G, Schena L, Castiglione V, Pentimone I, Nigro F, Silvestro Id y Ippolito A, 2007. Control of table grape storage rots by combined applications of antagonistic yeasts, salts and natural substances. *Proceedings of the International Congress Novel approaches for the control of postharvest diseases and disorders. Bologna, Italy:* 124-128.

Lima G, Ippolito A, Nigro F y Salerno M, 1997. Biological control of grey mould of stored table grapes by pre-harvest applications of *Aureobasidium pullulans* and *Candida oleophila*. *Difesa delle Piante*, **20**, 21-28.

Lima G, Castoria R, Spina AM, De Curtis F y Caputo L, 2005. Improvement of biocontrol yeast activity against postharvest pathogens: Recent experiences. En: Mencarelli F. and Tonutti P., eds. *Proceedings of the 5th International Postharvest Symposium, Vols 1-3.* 2035-2040.

Louis C, Girard M, Kuhl G y LopezFerber M, 1996. Persistence of *Botrytis cinerea* in its vector *Drosophila melanogaster*. *Phytopathology*, **86**, 934-939.

Magan N, 2001. Physiological approaches to improving the ecological fitness of fungal biocontrol agents. En: Butt T. M., Jackson C. and Magan N., eds. *Fungi as biocontrol agents: progress, problems and potential.* Oxon, UK: CABI Publishing, 239-251.

Martinez F, Blancard D, Lecomte P, Levis C, Dubos B y Fermaud M, 2003. Phenotypic differences between *vacuma* and *transposa* subpopulations of *Botrytis cinerea*. *European Journal of Plant Pathology*, **109**, 479-488.

Martinez F, Dubos B y Fermaud M, 2005. The role of saprotrophy and virulence in the population dynamics of *Botrytis cinerea* in vineyards. *Phytopathology*, **95**, 692-700.

Mehofer M, Hanak K, Diwald M y Regner F, 2009. Investigations into botrytis control in organic-biological viticulture. *Mitteilungen Klosterneuburg*, **59**, 189-198.

Mehofer M, 2011. Bunch Thinning by means of Gibberellins. *Mitteilungen Klosterneuburg*, **59**, 4-8.

Melgarejo-Flores BG, Ortega-Ramírez LA, Silva-Espinoza BA, González-Aguilar GA, Miranda MRA y Ayala-Zavala JF, 2013. Antifungal protection and antioxidant enhancement of table grapes treated with emulsions, vapors, and coatings of cinnamon leaf oil. *Postharvest Biology and Technology*, **86**, 321-328.

Mendoza L, Yanez K, Vivanco M, Melo R y Cotoras M, 2013. Characterization of extracts from winery by-products with antifungal activity against *Botrytis cinerea*. *Industrial Crops and Products*, **43**, 360-364.

Meng X y Tian S, 2009. Effects of preharvest application of antagonistic yeast combined with chitosan on decay and quality of harvested table grape fruit. *Journal of the Science of Food and Agriculture*, **89**, 1838-1842.

Meng XH, Li BQ, Liu J y Tian SP, 2008. Physiological responses and quality attributes of table grape fruit to chitosan preharvest spray and postharvest coating during storage. *Food Chemistry*, **106**, 501-508.

Ministerio de Agricultura PyA y Mundi-Prensa E, eds, 2004. *Los parásitos de la vid - Estrategias de protección razonada*. Madrid: Secretaría General Técnica. Centro de publicaciones. MAPA.

Mlikota F, Smilanick JL, Mansour M, Ramming DW y Mackey BE, 2003. Correlations of morphological, antomical, and chemical features of grapes berries with resistance to *Botrytis cinerea*. *Phytopathology*, **93**, 1263-1273.

Molitor D, Behr M, Fischer S, Hoffmann L y Evers D, 2011. Timing of cluster-zone leaf removal and its impact on canopy morphology, cluster structure and bunch rot susceptibility of grapes. *Journal International des Sciences de la Vigne et du Vin*, **45**, 149-159.

Mondy N, Pracros P, Fermaud M y Corio-Costet MF, 1998. Olfactory and gustatory behaviour by larvae of *Lobesia botrana* in response to *Botrytis cinerea*. *Entomologia Experimentalis et Applicata*, **88**, 1-7.

Montealegre JR, Lopez C, Stadnik MJ, Henriquez JL, Herrera R, Polanco R, Di Piero RM y Perez LM, 2010. Control of grey rot of apple fruits by biologically active natural products. *Tropical Plant Pathology*, **35**, 271-276.

Montesinos E, 2003. Development, registration and commercialization of microbial pesticides for plant protection. *International Microbiology*, **6**, 245-252.

Mundy DC y Agnew RH, 2002. Effects of mulching with vineyard and winery waste on soil fungi and botrytis bunch rot in Marlborough vineyards. En: Zydenbos S. M., ed. *New. Proceedings of a conference, Centra Hotel, Rotorua, New Zealand, 13-15 August 2002. New Zealand Plant Protection,* **55**, 135-138.

Mundy DC, Agnew RH y Wood PN, 2012. Grape tendrils as an inoculum source of *Botrytis cinerea* in vineyards - a review. *New Zealand Plant Protection*, **65**, 218-227.

Nair NG y Allen RN, 1993. Infection of grape flowers and berries by *Botrytis cinerea* as a function of time and temperature. *Mycological Research*, **97**, 1012-1014.

Nair NG, Guilbaud-Oulton S, Barchia I y Emmett R, 1995. Significance of carry over inoculum, flower infection and latency on the incidence of *Botrytis cinerea* in berries of grapevines at harvest in New South Wales. *Australian Journal of Experimental Agriculture*, **35**, 1177-1180.

Nally MC, Pesce VM, Maturano YP, Munoz CJ, Combina M, Toro ME, Castellanos de Figueroa LI y Vazquez F, 2012. Biocontrol of *Botrytis cinerea* in table grapes by non-pathogenic indigenous *Saccharomyces cerevisiae* yeasts isolated from viticultural environments in Argentina. *Postharvest Biology and Technology*, **64**, 40-48.

Nally MC, Pesce VM, Maturano YP, Toro ME, Combina M, Castellanos de Figueroa LI y Vazquez F, 2013. Biocontrol of fungi isolated from sour rot infected table grapes by *Saccharomyces* and other yeast species. *Postharvest Biology and Technology*, **86**, 456-462.

Nicot PC, ed, 2011. *Classical and Augmentative Biological Control Against Diseases and Pests: Critical Status Analysis and Review of Factors Influencing Their Success*. Avignon, France: IOBC/WPRS.

Nigro F, Schena L, Ligorio A, Pentimone I, Ippolito A y Salerno MG, 2006. Control of table grape storage rots by pre-harvest applications of salts. *Postharvest Biology and Technology*, **42**, 142-149.

Nunes C, Usall J, Teixidó N, Abadias M y Viñas I, 2001a. Improved control of postharvest decay of pears by the combination of *Candida sake* (CPA-1) and ammonium molybdate. *Phytopathology*, **92**, 281-287.

Nunes C, Usall J, Teixidó N, Miró M y Viñas I, 2001b. Nutritional enhancement of biocontrol activity of *Candida sake* (CPA-1) against *Penicillium expansum* on apples and pears. *European Journal of Plant Pathology*, **107**, 543-551.

Nunes C, Usall J, Teixidó N, Ochoa de Eribe X y Viñas I, 2001c. Control of post-harvest decay of apples by preharvest and postharvest application of ammonium molybdate. *Pest Management Science*, **57**, 1093-1099.

Nunes C, Usall J, Teixidó N y Viñas I, 2001d. Biological control of postharvest pear diseases using a bacterium *Pantoea agglomerans* CPA-2. *International Journal of Food Microbiology*, **70**, 53-61.

Nunes C, Usall J, Teixidó N, Torres R y Viñas I, 2002a. Control of *Penicillium expansum* and *Botrytis cinerea* on apples and pears with the combination of *Candida sake* and *Pantoea agglomerans*. *Journal of Food Protection*, **65**, 178-184.

Nunes C, Usall J, Teixidó N y Viñas I, 2002b. Improvement of *Candida sake* biocontrol activity against post-harvest decay by the addition of ammonium molybdate. *Journal of Applied Microbiology*, **92**, 927-935.

Oliva J, Navarro S, Barba A, Navarro G y Salinas MR, 1999. Effect of pesticide residues on the aromatic composition of red wines. *Journal of Agricultural and Food Chemistry*, **47**, 2830-2836.

Oliva J, Zalacain A, Paya P, Salinas MR y Barba A, 2008. Effect of the use of recent commercial fungicides [under good and critical agricultural practices] on the aroma composition of Monastrell red wines. *Analytica Chimica Acta*, **617**, 107-118.

Oriolani EJ, Rodriguez-Romera M y Combina M, 2009. Parasitic complex of acid rot bunches of grapes, in Mendoza and San Juan, Argentina En. *Abstract book of the XV Latin-American Congress and XVIII Chilean Congress of Plant Pathology, Santiago, Chile*, 204.

Palla O, 2006. A new formulation based on fatty acids coming from vegetal oils effective against grapevine downy mildew. En: Brunelli A., Canova A. and Collina M., eds. *Proceedings of the Giornate Fitopatologiche 2006. Riccione, Italia.* 17-20.

Palmer AK, Evans KJ y Metcalf DA, 2010. Characters of aerated compost tea from immature compost that limit colonization of bean leaflets by *Botrytis cinerea*. *Journal of Applied Microbiology*, **109**, 1619-1631.

Parry F, Elmer P, Wood P, Agnew R, Saunders J, Wurms K, Hoyte S, Chee A y Chee AA, 2011. Developing new biologically-based products for control of botrytis bunch rot. Part 2: Developing a new biologically-based product for late-season botrytis control: The BCA-L1 story. *Wine and Viticulture Journal*, **26**, 73-78.

Pavan F, Bigot G, Cargnus E y Zandigiacomo P, en prensa. Influence of the carpophagous generations of the European grapevine moth *Lobesia botrana* on grape bunch rots. *Phytoparasitica*, 1-9.

Petit AN, Vaillant-Gaveau N, Walker AS, Leroux P, Baillieul F, Panon ML, Clement C y Fontaine F, 2010. Determinants of fenhexamid effectiveness against grey mould on grapevine: Respective role of spray timing, fungicide resistance and plant defences. *Crop Protection*, **29**, 1162-1167.

Pezet R, Viret O, Perret C y Tabacchi R, 2003. Latency of *Botrytis cinerea* Pers.: Fr. and biochemical studies during growth and ripening of two grape berry cultivars, respectively susceptible and resistant to grey mould. *Journal of Phytopathology*, **151**, 208-214.

Qin GZ, Tian SP, Xu Y, Chan ZL y Li BQ, 2006. Combination of antagonistic yeasts with two food additives for control of brown rot caused by *Monilinia fructicola* on sweet cherry fruit. *Journal of Applied Microbiology*, **100**, 508-515.

Reglinski T, Elmer PAG, Taylor JT, Parry FJ, Marsden R y Wood PN, 2005. Suppression of *Botrytis* bunch rot in Chardonnay grapevines by induction of host resistance and fungal antagonism. *Australasian Plant Pathology*, **34**, 481-488.

Reglinski T, Elmer PAG, Taylor JT, Wood PN y Hoyte SM, 2010. Inhibition of *Botrytis cinerea* growth and suppression of botrytis bunch rot in grapes using chitosan. *Plant Pathology*, **59**, 882-890.

Romanazzi G, Nigro F, Ippolito A, Di Venere D y Salerno M, 2002. Effects of pre- and postharvest chitosan treatments to control storage grey mold of table grapes. *Food Microbiology and Safety*, **67**, 1862-1867.

Romanazzi G, Gabler FM y Smilanick JL, 2006. Preharvest chitosan and postharvest UV irradiation treatments suppress gray mold of table grapes. *Plant Disease*, **90**, 445-450.

Romanazzi G, Gabler FM, Margosan D, Mackey BE y Smilanick JL, 2009. Effect of chitosan dissolved in different acids on its ability to control postharvest gray mold of table grape. *Phytopathology*, **99**, 1028-1036.

Romanazzi G, Lichter A, Gabler FM y Smilanick JL, 2012. Recent advances on the use of natural and safe alternatives to conventional methods to control postharvest gray mold of table grapes. *Postharvest Biology and Technology*, **63**, 141-147.

Rooney-Latham S, Janousek CN y Gubler W, 2007. The use of various pre-harvest practices for the managment of "sour rot" and "Non-Botrytis Slip Skin" of Red Globe table grapes. *Phytopathology*, **97**, S101-S101.

Rosslenbroich HJ y Stuebler D, 2000. *Botrytis cinerea* - history of chemical control and novel fungicides for its management. *Crop Protection*, **19**, 557-561.

Sanzani SM, Schena L, De Cicco V y Ippolito A, 2012. Early detection of *Botrytis cinerea* latent infections as a tool to improve postharvest quality of table grapes. *Postharvest Biology and Technology*, **68**, 64-71.

Schena L, Nigro F, Pentimone I, Ligorio A y Ippolito A, 2003. Control of postharvest rots of sweet cherries and table grapes with endophytic isolates of *Aureobasidium pullulans*. *Postharvest Biology and Technology*, **30**, 209-220.

Schildberger B, Faltis C, Arnold M y Eder R, 2011. Effects of prohexadione-calcium on grape cluster structure and susceptibility to bunch rot (*Botrytis cinerea*) in cv. Gruner veltliner. *Journal of Plant Pathology*, **93**, 33-37.

Schilder AMC, Gillett JM, Sysak RW y Wise JC, 2002. Evaluation of environmentally friendly products for control of fungal diseases of grapes. En: Boos, Markus, eds. *10th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit-Growing and Viticulture. Proceedings to the Conference from 4th to 7th Feb. 2002 at Weinsberg/Germany*. 163-167.

Segarra G, Casanova E, Borrero C, Aviles M y Trillas I, 2007. The suppressive effects of composts used as growth media against *Botrytis cinerea* in cucumber plants. *European Journal of Plant Pathology*, **117**, 393-402.

Servei de Sanitat Vegetal, 2002. Plagues: La podridura grisa (*Botrytis cinerea*). En: *Catalunya Rural i Agrària.* DAAM, **91,** 39-40. Online (http://www20.gencat.cat/docs /DAR/AG_Agricultura/AG02_Sanitat_vegetal/AG02_02_Plagues_males_herbes/Enllacos_interns/FITXA_43_Botrytis_vinya.pdf, Acceso 31/10/2013)

Seyb A, 2004. *Botrytis cinerea inoculum sources in the vineyard system*. Cristchurch: Lincoln University. PhD thesis

Sharma RR, Singh D y Singh R, 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. *Biological Control*, **50**, 205-221.

Shirane N y Watanabe Y, 1985. Comparison of infection process of *Botrytis cinerea* on cucumber cotyledon and strawberry petal. *Annals of the Phytopathological Society of Japan*, **51**, 501-505.

Sicher L, Dorigoni A y Stringari G, 1995. Soil management effects on nutritional status and grapevine performance. En: Tagliavini M., Neilsen G. H. and Millard P., eds. *Acta Horticulturae.* 73-82.

Spadaro D y Gullino ML, 2004. State of the art and future prospects of the biological control of postharvest fruit diseases. *International Journal of Food Microbiology.* **91,** 185-194.

Spadaro D y Gullino ML, 2010. Opportunities and constraints in the development of antagonistic yeasts for the control of postharvest diseases of fruit. *Stewart Postharvest Review*, **6**, 1-8.

Spring JL y Viret O, 2011. Influence of thinning methods on yield, bunch morphology and sensitivity against grey mould on the grape variety Gamay. *Revue Suisse de Viticulture, Arboriculture et Horticulture*, **43**, 280-287.

Stapleton JJ y Grant RS, 1992. Leaf removal for nonchemical control of the summer bunch rot complex of wine grapes in the San-Joaquin valley. *Plant Disease*, **76**, 205-208.

Steel CC y Greer DH, 2008. Effect of climate on vine and bunch characteristics: Bunch rot disease susceptibility. En: Adsule P. G., Sawant I. S. and Shikhamany S. D., eds. *Proceedings of the International Symposium on Grape Production and Processing. Acta Horticulturae,* **785,** 253-262.

Steel CC, Greer DH, Samuelian SK y Savocchia S, 2010. Effects of climate and mixed bunch rot infection on *Botrytis cinerea* (grey mould) of grapes: Implications for global climate change and disease management. En: *Abstract book of the XV International Botrytis symposium.* Cadiz, Spain, 57.

Steel CC, Greer LA, Savocchia S y Samuelian SK, 2011. Effect of temperature on *Botrytis cinerea*, /*Colletotrichum acutatum* and *Greeneria uvicola* mixed fungal infection of *Vitis vinifera* /grape berries. *Vitis*, **50**, 69-71.

Steel CC, Blackman JW y Schmidtke LM, 2013. Grapevine bunch rots: Impacts on wine composition, quality, and potential procedures for the removal of wine faults. *Journal of Agricultural and Food Chemistry*, **61**, 5189-5206.

Suarez-Estrella F, Jurado MM, Vargas-Garcia MC, Lopez MJ y Moreno J, 2013. Isolation of bio-protective microbial agents from eco-composts. *Biological Control*, **67**, 66-74.

Tardaguila J, Blanco JA, Poni S y Diago P, 2012. Mechanical yield regulation in winegrapes: comparison of early defoliation and crop thinning. *Australian Journal of Grape and Wine Research*, **18**, 344-352.

Teixidó N, Usall J, Gutierrez O y Viñas I, 1998a. Effect of the antagonist *Candida sake* on apple surface microflora during cold and ambient (shelf life) storage. *European Journal of Plant Pathology*, **104**, 387-398.

Teixidó N, Viñas I, Usall J y Magan N, 1998b. Control of blue mold of apples by preharvest application of *Candida sake* grown in media with different water activity. *Phytopatology*, **88**, 960-964.

Teixidó N, Viñas I, Usall J y Magan N, 1998c. Improving ecological fitness and environmental stress tolerance of the biocontrol yeast *Candida sake* by manipulation of intracellular sugar alcohol and sugar content. *Mycological Research*, **102**, 1409-1417.

Teixidó N, Viñas I, Usall J, Sanchis V y Magan N, 1998d. Ecophysiological responses of the biocontrol yeast *Candida sake* to water, temperature and pH stress. *Journal of Applied Microbiology*, **84**, 192-200.

Teixidó N, Usall J y Viñas I, 1999. Efficacy of preharvest and postharvest *Candida sake* biocontrol treatments to prevent blue mould on apples during cold storage. *International Journal of Food Microbiology*, **50**, 203-210.

Teixidó N, Usall J, Palou L, Asensio A, Nunes C y Viñas I, 2001. Improving control of green and blue molds of oranges by combining *Pantoea agglomerans* (CPA-2) and sodium bicarbonate. *European Journal of Plant Pathology*, **107**, 685-694.

Teixidó N, Usall J, Nunes C, Torres R, Abadias M y Vinas I, 2010. Preharvest strategies to control postharvest diseases in fruits. En: Prusky D. and Gullino M. L., eds. *Postharvest Pathology* (Plant Pathology in the 21st Century; vol. 2). Springer Netherlands, 89-106.

Teixidó N, Torres R, Viñas I, Abadias M y Usall J, 2011. Biological control of postharvest diseases in fruit and vegetables. En: Lacroix C., ed. *Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage* (Food Science, Technology and Nutrition vol. **3**). Cambridge, UK: Woodhead Publishing Limited, 364-402.

Termorshuizen AJ, van Rijn E, van der Gaag DJ, Alabouvette C, Chen Y, Lagerlof J, Malandrakis AA, Paplomatas EJ, Ramert B, Ryckeboer J, Steinberg C y Zmora-Nahum S, 2006. Suppressiveness of 18 composts against 7 pathosystems: Variability in pathogen response. *Soil Biology & Biochemistry*, **38**, 2461-2477.

Tesfagiorgis HB y Annegarn HJ, 2013. Evaluation of adjuvants for enhanced management of *Podosphaera xanthii* using potassium silicate and biocontrol agents. *Crop Protection*, **48**, 69-75.

Tian SP, Qin GZ y Xu Y, 2004. Survival of antagonistic yeasts under field conditions and their biocontrol ability against postharvest diseases of sweet cherry. *Postharvest Biology and Technology*, **33**, 327-331.

Torres R, Usall J, Teixidó N, Abadias M y Viñas I, 2003. Liquid formulation of the biocontrol agent *Candida sake* by modifying water activity or adding protectants. *Journal of Applied Microbiology*, **94**, 330-339.

Torres R, Teixidó N, Viñas I, Mari M, Casalini L, Giraud M y Usall J, 2006. Efficacy of *Candida sake* CPA-1 formulation in controlling *Penicillium expansum* decay on pome fruit from different mediterranean regions. *Journal of Food Protection*, **69**, 2703-2711.

Tripathi P y Dubey NK, 2004. Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biology and Technology*, **32**, 235-245.

Urbaso S, 2003. Documento técnico 11/5 - Modelo para el seguimiento del Oidio y la *Botrytis* en la vid. Online (http://www.urbaso.com/dokutek/HToidio_botritis.PDF; Acceso 31/10/2013).

Usall J, 1995. *Control biològic de Penicillium expansum en postcollita de fruita de llavor*. Lleida: Universitat de Lleida, PhD Thesis.

Usall J, Teixidó N, Fons E y Viñas I, 2000. Biological control of blue mould on apple by a strain of *Candida sake* under several controlled atmosphere conditions. *International Journal of Food Microbiology*, **58**, 83-92.

Usall J, Teixidó N, Torres R, Ochoa de Eribe X y Viñas I, 2001. Pilot tests of *Candida sake* (CPA-1) applications to control postharvest blue mold on apple fruit. *Postharvest Biology and Technology*, **21**, 147-156.

Valdés-Gómez H, Fermaud M, Roudet J, Calonnec A y Gary C, 2008. Grey mould incidence is reduced on grapevines with lower vegetative and reproductive growth. *Crop Protection*, **27**, 1174-1186.

Valero D, Valverde JM, Martinez-Romero D, Guillen F, Castillo S y Serrano M, 2006. The combination of modified atmosphere packaging with eugenol or thymol to maintain quality, safety and functional properties of table grapes. *Postharvest Biology and Technology*, **41**, 317-327.

van Kan JAL, 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends in Plant Science*, **11**, 247-253.

Vargas M, Garrido F, Zapata N y Tapia M, 2012. Isolation and selection of epiphytic yeast for biocontrol of *Botrytis cinerea* pers. on table grapes. *Chilean Journal of Agricultural Research*, **72**, 332-337.

Vercesi A, Pontiroli R y Rizzotti R, 2001. Organic viticulture and control. *Vignevini*, **28**, 55-62.

Viñas I, Usall J, Teixidó N y Sanchis V, 1998. Biological control of major postharvest pathogens on apple with *Candida sake*. *International Journal of Food Microbiology*, **40**, 9-16.

Vitoratos A, Bilalis D, Karkanis A y Efthimiadou A, 2013. Antifungal activity of plant essential oils against *Botrytis cinerea*, *Penicillium italicum* and *Penicillium digitatum*. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, **41**, 86-92.

Vivier MA y Pretorius IS, 2002. Genetically tailored grapevines for the wine industry. *Trends in Biotechnology*, **20**, 472-478.

Walker A-S, Micoud A, Remuson F, Grosman J, Gredt M y Leroux P, en prensa. French vineyards provide information that opens ways for effective resistance management of *Botrytis cinerea* (grey mould). *Pest Management Science*, **69**, 667-678.

Walter M, Jaspers MV, Eade K, Frampton CM y Stewart A, 2001. Control of *Botrytis cinerea* in grape using thyme oil. *Australasian Plant Pathology*, **30**, 21-25.

Wei Y, Li X, Cai J, Zhang X, Liu Z, Shang Q y Zhao X, 2011. Identification of yeasts associated with grape sour rot in the north of China. *Phytopathology*, **101**, S189-S189.

Whipps J y Lumsden R, 2001. Commercial use of fungi as plant disease biological control agents: status and prospects. En: Butt T. M., Jackson C. and Magan N., eds. *Fungi as biocontrol agents: progress, problems and potential.* Oxon, UK: CABI Publishing, 9-22.

Williamson B, Tudzynski B, Tudzynski P y van Kan JAL, 2007. *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology*, **8**, 561-580.

Wolf TK, Baudoin A y Martinez-Ochoa N, 1997. Effect of floral debris removal from fruit clusters on botrytis bunch rot of Chardonnay grapes. *Vitis*, **36**, 27-33.

Wurms K, Chee A, Elmer P, Agnew R, Wood P y Chee AA, 2011. Developing new biologically-based products for control of botrytis bunch rot. Part 1: Developing a new natural product for mid-season botrytis control - NP2 moves closer to the market. *Wine and Viticulture Journal*, **26**, 64-72.

Xu WT, Huang KL, Guo F, Qu W, Yang JJ, Liang ZH y Luo YB, 2007. Postharvest grapefruit seed extract and chitosan treatments of table grapes to control *Botrytis cinerea*. *Postharvest Biology and Technology*, **46**, 86-94.

Yan H, Yan J, Wang Z, Li Y, Jin G y Li X, 2012. Multiple fungicide resistance of *Botrytis cinerea* from grapevine to three fungicides. *Journal of Fruit Science*, **29**, 625-629.

Yánez–Mendizábal V, Usall J, Viñas I, Casals C, Marín S, Solsona C y Teixidó N, 2010. Potential of a new strain of *Bacillus subtilis* CPA–8 to control the major postharvest diseases of fruit. *Biocontrol Science and Technology*, **21**, 409-426.

Younglai EV, Wu YJ y Foster WG, 2007. Reproductive toxicology of environmental toxicants: Emerging issues and concerns. *Current Pharmaceutical Design*, **13**, 3005-3019.

Youssef K y Roberto SR, 2014. Applications of salt solutions before and after harvest affect the quality and incidence of postharvest gray mold of 'italia' table grapes. *Postharvest Biology and Technology*, **87**, 95-102.

Zahavi T, Cohen L, Weiss B, Schena L, Daus A, Kaplunov T, Zutkhi J, Ben-Arie R y Droby S, 2000. Biological control of *Botrytis*, *Aspergillus* and *Rhizopus* rots on table and wine grapes in Israel. *Postharvest Biology and Technology*, **20**, 115-124.

Zhang H, Li R y Liu W, 2011. Effects of chitin and its derivative chitosan on postharvest decay of fruits: a review. *International Journal of Molecular Sciences*, **12**, 917-934.

Zhao H, Kim YK, Huang L y Xiao CL, 2010. Resistance to thiabendazole and baseline sensitivity to fludioxonil and pyrimethanil in *Botrytis cinerea* populations from apple and pear in Washington State. *Postharvest Biology and Technology*, **56**, 12-18.

Zoecklein BW, Wolf TK, Duncan NW, Judge JM y Cook MK, 1992. Effects of fruit zone leaf removal on yield, fruit composition, and fruit rot incidence of Chardonnay and white Riesling (*Vitis vinifera* L.) grapes. *American Journal of Enology and Viticulture*, **43**, 139-148.

OBJETIVOS

OBJETIVOS

C. sake CPA-1 ha mostrado un gran potencial como ACB en estudios previos y se han completado numerosas etapas en su desarrollo para su comercialización como producto comercial. Además, los estudios previos en viña mostraron alta efectividad en el control de la PBC en viña, una enfermedad importante económicamente y para la que existen escasos tratamientos alternativos a los fungicidas de síntesis.

El objetivo principal de esta tesis es, por tanto, confirmar la eficacia de los tratamientos con *C. sake* y FC observada en campo, evaluándola bajo diferentes condiciones climáticas, en viñedos de manejo ecológico y convencional, comparando con otros tratamientos biológicos e intentando optimizar su dosis y momento de aplicación. Así mismo, el conocimiento de la epidemiología de la enfermedad es un elemento importante para maximizar la eficacia de los tratamientos, mientras que el grado de supervivencia del ACB sobre las superficies de la planta es uno de los factores que más comprometen su efectividad. Por este motivo, diversos aspectos de la epidemiología de la PBC y de la supervivencia de *C. sake* ante los factores abióticos que la determinan fueron igualmente evaluados.

A continuación se detallan los objetivos específicos de la presente tesis:

1. Estudiar la eficacia de *C. sake* CPA-1 con el aditivo Fungicover en el control de la podredumbre por *B. cinerea* en ensayos de campo y laboratorio

1.1. Estudiar la efectividad de las aplicaciones de *C. sake* y FC en campo, en viñedos de manejo ecológico y convencional, en varias campañas y en condiciones de clima mediterráneo y atlántico
1.2. Comparar su efectividad con otros productos registrados y en uso para el control de la PBC (quitosán y *U. oudemansii*)
1.3. Optimizar los tratamientos de campo con *C. sake* y FC mediante la evaluación de diferentes dosis y calendarios de aplicación
1.4. Evaluar el efecto de estos tratamiento biológicos sobre las fuentes de inóculo secundario en tejidos necróticos del interior del racimo (caliptras, flores abortadas y frutos abortados)
1.5. Comprobar la efectividad de *C. sake* y FC en ensayos de laboratorio con diferentes cepas virulentas de *B. cinerea*
1.6. Estudiar la efectividad y el modo acción del producto basado en ácidos grasos FC, comparando con el producto comercial PRT

2. Evaluar el efecto de estos tratamientos de campo sobre la podredumbre ácida del racimo

2.1. Evaluar la reducción de la incidencia y severidad de la podredumbre ácida en viñas tratadas con *C. sake* y FC y otros tratamientos de origen biológico
2.2. Caracterizar la relación de los niveles de incidencia y severidad de esta enfermedad respecto a los de la PBC y su hipotética asociación con las condiciones meteorológicas durante la campaña

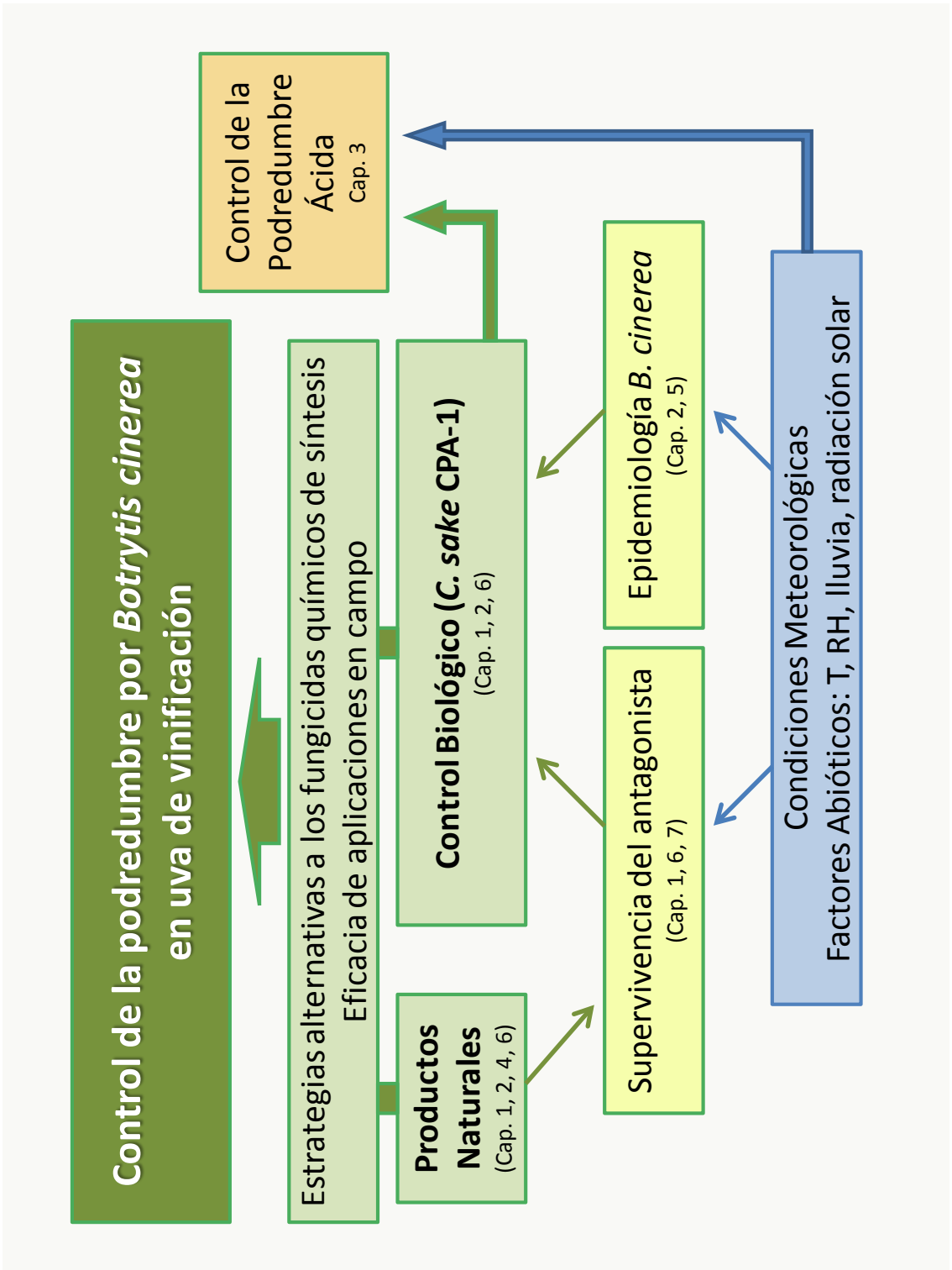
3. Investigar la epidemiología de la PBC en viñedos de climatología mediterránea (Lleida)

3.1. Determinar los momentos más importantes durante la campaña para el desarrollo de la incidencia y severidad de la PBC en momento de cosecha
3.2. Determinar los estados fenológicos más importantes durante la primera parte de la campaña para el desarrollo de diferentes fuentes de inóculo secundario de *B. cinerea* (infecciones latentes, tejidos necróticos del racimo e inóculo superficial)
3.3. Cuantificar la incidencia de *B. cinerea* en los diferentes tejidos necróticos del interior del racimo en momento de envero (caliptras, flores abortadas y frutos abortados)
3.4. Estudiar cuantitativamente la posible correlación entre las fuentes de inóculo secundario y la PBC en cosecha

4. Evaluar el efecto de los factores que reducen la supervivencia de *C. sake* CPA-1 en campo: Temperatura, Humedad Relativa, lluvia y radiación solar

4.1. Describir la dinámica poblacional de las poblaciones de *C. sake* CPA-1 en las aplicaciones de campo realizadas para el control de la PBC, y su relación con el control obtenido
4.2. Evaluar la supervivencia de *C. sake* aplicada con FC bajo condiciones limitantes de T y HR, en condiciones controladas en cámaras climáticas y en condiciones externas de verano en clima mediterráneo
4.3. Estudiar el efecto protector del aditivo FC sobre las poblaciones de *C. sake* expuestas a condiciones limitantes
4.4. Cuantificar el efecto del deshojado y la consecuente exposición de los racimos a los factores abióticos sobre las poblaciones de *C. sake* en campo
4.5. Determinar la persistencia de *C. sake* aplicada con FC en uvas expuestas a lluvia simulada, aplicando diferentes volúmenes e intensidades de lluvia
4.6. Evaluar el efecto de un periodo de incubación para favorecer el establecimiento de *C. sake*, previo a un periodo de lluvia simulada o condiciones limitantes de T y HR, en la reducción de las poblaciones del ACB en uvas

ESTRUCTURA DE LOS ESTUDIOS REALIZADOS


Control de la podredumbre por Botrytis cinerea en uva de vinificación
Estrategias alternativas a los fungicidas químicos de síntesis
Eficacia de aplicaciones en campo
Control de la Podredumbre Ácida
Cap. 3
Control Biológico (C. sake CPA-1)
(Cap. 1, 2, 6)
Productos Naturales
(Cap. 1, 2, 4, 6)
Epidemiología B. cinerea
(Cap. 2, 5)
Supervivencia del antagonista
(Cap. 1, 6, 7)
Condiciones Meteorológicas
Factores Abióticos: T, RH, lluvia, radiación solar

CAPÍTULO 1

Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1

C. Calvo-Garrido, P. A. G. Elmer, I. Viñas, J. Usall, E. Bartra and N. Teixidó

Plant Pathology **(2013), 62, 510–519**

Plant Pathology (2013) **62,** 510–519 Doi: 10.1111/j.1365-3059.2012.02684.x

Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1

C. Calvo-Garrido[a], P. A. G. Elmer[b], I. Viñas[a], J. Usall[c], E. Bartra[d] and N. Teixidó[c]*

[a]*University of Lleida, XaRTA-Postharvest, 191 Rovira Roure, 25198, Lleida, Catalonia, Spain;* [b]*The New Zealand Institute for Plant & Food Research Limited, Ruakura Research Centre, Private Bag 3230, Waikato Mail Centre, 3240, Hamilton, New Zealand;* [c]*IRTA, XaRTA-Postharvest, 191 Rovira Roure, 25198, Lleida, Catalonia, Spain; and* [d]*INCAVI, Pl. Ágora 2–3, 08720, Vilafranca del Penedes, Catalonia, Spain*

The aim of this research was to confirm the efficacy of the yeast antagonist *Candida sake* CPA-1 in suppressing botrytis bunch rot development, in an organic vineyard under Mediterranean conditions for two seasons, and compare its performance with that of two biologically based products currently registered for botrytis bunch rot control in New Zealand. In 2009, treatments applied were: commercial formulations of *Ulocladium oudemansii* (BOTRY-Zen®) and chitosan (ARMOUR-Zen®), *C. sake* CPA-1 combined with the fatty acid-based additive Fungicover® and combinations of these products. All treatments were applied six times between early flowering and harvest and compared with an unsprayed control. In 2010, the treatments focused on *C. sake* and Fungicover and the number of applications was reduced from six to four. The population dynamics of *U. oudemansii* and *C. sake* were measured and wine quality tests were carried out in both seasons. Disease control achieved by *C. sake* treatments in 2009 were comparable to those achieved by BOTRY-Zen and ARMOUR-Zen. Applications of *C. sake* plus Fungicover between flowering and harvest significantly ($P < 0{\cdot}05$) reduced botrytis bunch rot incidence and severity by 64% and 90%, respectively, compared with the untreated control in 2009, and by 67% and 89%, respectively, in 2010. Treatments did not adversely affect wine quality parameters after treated grapes were processed. *Candida sake* consistently provided effective control of botrytis bunch rot in grapes under different meteorological and disease pressure conditions, thereby improving its potential for future commercial applications.

Keywords: ARMOUR-Zen® chitosan, BOTRY-Zen®, Fungicover®, microvinification, population dynamics, *Ulocladium oudemansii*

Introduction

The necrotrophic pathogen *Botrytis cinerea* is a filamentous fungus of the Sclerotiniaceae family that is able to infect a broad range of hosts (Holz *et al.*, 2004). In grapevines it is responsible for botrytis bunch rot or grey mould and in many temperate regions it is the most important fungal disease that affects grape production before harvest and wine quality postharvest (Elmer & Reglinski, 2006). Conventional approaches to botrytis bunch rot control have focused on synthetic fungicide applications. However, sole reliance upon this approach is not sustainable because of the emergence of fungicide resistance in vineyard populations of *B. cinerea* (Leroch *et al.*, 2011) and the adverse effects of synthetic pesticides on environmental and human health (Komárek *et al.*, 2010). Increasing consumer demand for no detectable pesticide residues in wine has forced many producers to restrict the application of synthetic fungicides to the early part of the growing season, thereby reducing the risk of residue being detected in the wine (Elmer & Michailides, 2004). Unfortunately, this strategy is flawed because rapidly ripening berries in the post-véraison period are highly susceptible to infection by *B. cinerea* (Hill *et al.*, 1981) and in the absence of a suitable protectant, significant crop losses may occur. Substituting synthetic fungicides in the mid- and late season with commercially available biologically based products has been reported to provide acceptable control of botrytis bunch rot in grapes, indicating that there is potential to integrate early season synthetic fungicides with biologically based products after bunch closure (Parry *et al.*, 2011).

In line with low pesticide strategies, organic viticulture continues to increase globally. The area of grapes grown organically in Catalonia increased by 140% between 2008 and 2010, while in Spain an overall increase of 5% of the area devoted to organically grown grapes was registered in the 2009–2010 period (MARM, 2010), further highlighting the importance of this sector of production in the future.

In organic viticulture, botrytis bunch rot control is usually carried out through cultural methods and canopy management, while other organically acceptable methods include spray applications of bentonite clays, copper-based formulations, compost teas or plant

*E-mail: neus.teixido@irta.cat

Published online 29 August 2012

extracts (Jacometti *et al.*, 2010). Botrytis bunch rot control based upon these strategies may be variable or have undesirable consequences on the aromatic compounds of some varieties (Jackson, 2008). These issues further highlight the urgent need for new organically acceptable full-season treatments for botrytis bunch rot control to complement canopy management and cultural practices.

A diverse range of alternative strategies has been evaluated by many research groups worldwide with special emphasis on natural products, elicitors of host defences and antimicrobial antagonists. Recent reviews of these alternative strategies include those by Elmer & Reglinski (2006) and Jacometti *et al.* (2010). A natural product that has shown promise against *B. cinerea* in grapes is chitosan: a naturally occurring polysaccharide derived from chitin. Efficacy studies on grapes have focused on postharvest treatment of table grapes (Xu *et al.*, 2007; Romanazzi *et al.*, 2009). Relatively fewer studies have focused on preharvest field applications to wine grapes, with the exception of Mehofer *et al.* (2009) and Reglinski *et al.* (2010). A chitosan-based product has been commercialized in New Zealand (ARMOUR-Zen®) with a label claim for botrytis bunch rot control in wine grapes (Reglinski *et al.*, 2010). Biological control of *B. cinerea* in grapes using naturally occurring microbial antagonists has been widely studied and several products have been developed and commercialized (Elmer & Reglinski, 2006; Jacometti *et al.*, 2010). An isolate of *Ulocladium oudemansii* was formulated and commercialized (BOTRY-Zen®), showing efficacy against botrytis bunch rot when applied during the early part of the growing season in New Zealand vineyards (Reglinski *et al.*, 2010; Wurms *et al.*, 2011).

Neither ARMOUR-Zen nor BOTRY-Zen has been evaluated under the hot and dry environmental conditions that occur during parts of the growing season in Catalan vineyards.

Yeasts have also been evaluated as potential biological control agents (BCAs) for *B. cinerea* (Elad & Stewart, 2004). The most important genera include *Pichia*, *Candida*, *Metschnikowia* (Jacometti *et al.*, 2010) and, more recently, *Saccharomyces cerevisiae* (Nally *et al.*, 2012). In field and laboratory studies, pre- and postharvest applications of *Candida* spp. have given effective control of *B. cinerea* in a wide range of crops; for example, *C. oleophila* (El-Neshawy & El-Morsy, 2003) and *C. guillermondii* (Scherm *et al.*, 2003) when applied to apples and *C. saitoana* (El Ghaouth *et al.*, 2003) for postharvest disease control in apples and citrus.

Candida sake CPA-1 is a yeast strain formulated and optimized by the postharvest pathology research group at IRTA Lleida. It was effective against postharvest *B. cinerea* in pome fruits (Torres *et al.*, 2006), suggesting its potential application against *B. cinerea* on other crops, such as grapes.

A previous study (Cañamás *et al.*, 2011) evaluated the effectiveness of *C. sake* CPA-1 against botrytis bunch rot applied under dry Mediterranean conditions (Catalonia, Spain) in conventional vineyards. Heat-adapted and non-adapted fresh cells were tested in different formulations with an isotonic solution or with the additive Fungicover. Fungicover was already known to be a beneficial additive to *C. sake*, improving the persistence of the BCA on the host and its efficacy to levels comparable to that of fungicide treatment. Thus, the application of *C. sake* CPA-1 and Fungicover as a treatment mixture was regarded as an interesting strategy to be further investigated in field trials.

This study was undertaken over two consecutive growing seasons in the Lleida winegrowing area (Catalonia, Spain). The aim was to confirm the effectiveness of field applications of treatments with *C. sake* CPA-1 plus Fungicover against botrytis bunch rot in organic vineyards, at different doses and numbers of applications, and compare their efficacy with that of other registered biological products. Treated vines were also processed into wine using microvinification techniques, to evaluate possible adverse effects of BCA treatments on wine quality.

Materials and methods

Microbial antagonists and natural products

Candida sake strain CPA-1 was isolated from the surface of apples at the University of Lleida-IRTA research centre and was deposited in the Colección Española de Cultivos Tipo (CECT-10817) at the University of Valencia, Burjassot, Spain. Cell production and formulation prior to field application was carried out according to methods described by Cañamás *et al.* (2011).

Ulocladium oudemansii isolate HRU3 was applied during flowering and up to pre-bunch closure using the formulated product BOTRY-Zen® (Botry-Zen Ltd). Chitosan was applied using the formulated product ARMOUR-Zen® (Botry-Zen Ltd). All treatments with BOTRY-Zen and ARMOUR-Zen included the wetting agent Mojante-Inagra (Sipcam Inagra S.A.) at 0·5 mL L^{-1}. ARMOUR-Zen treatments were also supplemented with 1·05 g L^{-1} sodium bicarbonate in order to adjust the final solution to pH 7, because studies indicated that this adjustment improved *B. cinerea* suppression *in vitro* (T. Reglinski, Plant and Food Research, New Zealand, personal communication).

Fungicover (BioDúrcal S.L.) is a commercial formulation of derivatives of fatty acids in an aqueous-alcoholic solution and previous research showed that this additive improved *C. sake* survival on grape host tissues (Cañamás *et al.*, 2011). Its use is authorized in Spain for conventional and organic agriculture by AGROCOLOR S.A., authorized agency under the Council Regulation (EEC) No. 2092/91, as a protector for preventing fungal diseases on a variety of crops (BioDúrcal, 2010).

Experimental field site (2009 and 2010)

Field experiments in both growing seasons were carried out in a commercial organic vineyard certified by the Catalan committee for organic agriculture production (CCPAE) and located in the Designation of Origin Costers del Segre (Lleida, Spain). The experimental site was located in a part of the vineyard with a history of botrytis bunch rot and trained into a bush system. The grape cultivar used was Macabeu, characterized by large and very compact clusters and thick-skinned berries (Fuster, 2006). These characteristics are reported to increase susceptibility to *B. cinerea*

(Fermaud *et al.*, 2001). Vines were sprayed with organically acceptable treatments throughout both growing seasons by the vineyard manager for pests and diseases. In 2009 these treatments consisted of three applications of 99% sulphur (w/w) prior to flowering and one more application of 99% sulphur (w/w) plus 98% silicon (w/w) at pre-bunch closure; in addition, one application of *Bacillus thuringiensis* var. *kurstaki* was made at véraison. In 2010, treatments included one application of 99% sulphur (w/w) prior to flowering, two applications of 60% sulphur plus 4% copper oxychloride at pre-bunch closure, one application of 38% copper oxychloride (w/v) at pre-bunch closure and two applications of *B. thuringiensis* var. *kurstaki* during véraison. All products were applied at the doses and application rates recommended by their manufacturers.

Experimental design

Plots were distributed in a completely randomized block design, with four replicates per treatment. Each plot consisted of seven vines; the first and last vines were used as buffer vines, the next two to monitor BCA population dynamics and the last three to measure botrytis bunch rot development.

All treatments were applied at key vine phenological stages (1–5% flowering, 80% flowering, pre-bunch closure, véraison, 21 and 7 days before harvest) using a motorized backpack sprayer (model WJR2225; Honda Motor Company Ltd) with a 1-mm nozzle and 15 bar pressure, spraying each grape bunch until run-off.

Bunch rot management programmes

The botrytis bunch rot management programmes used in 2009 are detailed in Table 1.

Final concentrations of the active ingredients applied were: chitosan 1·44 g L^{-1}, *U. oudemansii* $2{\cdot}5 \times 10^6$ colony-forming units (CFU) mL^{-1} and *C. sake* 5×10^7 CFU mL^{-1}. An additional treatment tested a reduced concentration of *C. sake*, 1×10^7 CFU mL^{-1}, in order to improve the potential economics of *C. sake* as a new BCA for application in vineyards. Fungicover® was applied at a dose of 50 g L^{-1} for all *C. sake* treatments.

In 2010, nine botrytis bunch rot control programmes were applied (Table 2), focusing on *C. sake* CPA-1 at 5×10^7 CFU mL^{-1} combined with Fungicover at different doses (50 or 25 g L^{-1}) and different application timings. Treatment applications were reduced to four or two, in order to evaluate whether more cost-effective spray programmes were achievable. Application methods and formulations of *C. sake* CPA-1 and Fungicover were the same as in 2009.

Botrytis bunch rot assessment

At harvest (14 September 2009 and 15 September 2010) botrytis bunch rot incidence and severity were assessed on 50 bunches per replicate plot. Twenty-five bunches were selected from each side of the row, avoiding vines used for population dynamics studies and buffer vines. Incidence was measured as the percentage of bunches with visual *B. cinerea* infection symptoms. Bunch rot severity was measured as the percentage of *B. cinerea*-infected berries per bunch.

Meteorological data

Temperature (T) and relative humidity (RH) were logged at hourly intervals in both seasons using a weather station (Decagon Services Inc.) placed at the experimental field site.

Population dynamics of biological control agents (2009 and 2010)

Populations of *C. sake* or *U. oudemansii* were measured in the 2009 and 2010 seasons by sampling treated flowers or berries (i) once the tissue surface was dry, just after treatment application, and (ii) before the next treatment application. Sampling of host tissues was carried out using sterile clippers with each plot sample placed directly into 50-mL sterile plastic tubes (Corning Inc.) that were then cool-stored prior to tissue processing on the same day. At flowering, BCA populations were recovered from 2 g floral organs from eight bunches per plot, collecting two groups of approximately 10 flowers per bunch. Samples where then immersed in 20 mL phosphate buffer in 250-mL conical flasks. At pre-bunch closure 40 pea-sized berries from 20 bunches per plot were weighed and then immersed in 50 mL phosphate buffer in 250-mL conical flasks. After véraison, 20

Table 1 Biologically based botrytis bunch rot control programmes applied to Macabeu wine grapes and botrytis bunch rot at harvest in an organic vineyard in 2009

	Vine phenology						Botrytis bunch rot at harvest[b]	
Treatment[a]	1–5% Flowering	80% Flowering	Pre-bunch closure	Véraison	21 days before harvest	7 days before harvest	Incidence (%)	Severity (%)
Control	–	–	–	–	–	–	80·0a	8·2a
CS + FC	CS + FC	CS + FC	CS + FC	CS + FC	CS + FC	CS + FC	28·5c	0·8b
CS low + FC	CS low + FC	CS low + FC	CS low + FC	CS low + FC	CS low + FC	CS low + FC	43·5b	1·9b
AZ	AZ	AZ	AZ	AZ	AZ	AZ	38·0bc	2·2b
BZ-AZ	BZ	BZ	BZ	AZ	AZ	AZ	40·5bc	1·7b
BZ-(CS + FC)	BZ	BZ	BZ	CS + FC	CS + FC	CS + FC	39·0bc	1·4b
(CS + FC)-AZ	CS + FC	CS + FC	CS + FC	AZ	AZ	AZ	30·0bc	1·2b
FC	FC	FC	FC	FC	FC	FC	39·0bc	1·5b

[a]Control: untreated; CS + FC: *Candida sake* at 5×10^7 colony-forming units (CFU) mL^{-1} + Fungicover at 50 g L^{-1}; CS low + FC: *C. sake* at 1×10^7 CFU mL^{-1} + Fungicover at 50 g L^{-1}; AZ: chitosan at 1·44 g L^{-1} + wetting agent at 0·5 mL L^{-1} + $NaHCO_3$ at 1·05 g L^{-1}; BZ: *Ulocladium oudemansii* at $2{\cdot}5 \times 10^6$ CFU mL^{-1} + wetting agent at 0·5 mL L^{-1}; FC: Fungicover at 50 g L^{-1}.

[b]All values are means of four replicates. Mean values with the same letter are not significantly different ($P = 0{\cdot}05$) according to LSD Student's *t*-test.

Table 2 *Candida sake* CPA-1 and Fungicover botrytis bunch rot control programmes applied to Macabeu wine grapes and botrytis bunch rot at harvest in an organic vineyard in 2010

	Vine phenology				Botrytis bunch rot at harvest[b]	
Treatment[a]	80% Flowering	Pre-bunch closure	Véraison	21 days before harvest	Incidence (%)	Severity (%)
Control	–	–	–	–	89·5a	21·7a
CS + FC × 4	CS + FC	CS + FC	CS + FC	CS + FC	29·5e	2·3b
FC × 4	FC	FC	FC	FC	55·0bcd	5·6b
CS + FC × 2	CS + FC	CS + FC	–	–	44·0d	3·2b
FC × 2	FC	FC	–	–	59·5bc	4·6b
CS + FC25 × 4	CS + FC25	CS + FC25	CS + FC25	CS + FC25	46·5d	3·3b
FC25 × 4	FC25	FC25	FC25	FC25	59·5bc	3·8b
CS + FC25 × 2	CS + FC25	CS + FC25	–	–	48·5cd	3·9b
FC25 × 2	FC25	FC25	–	–	62·5bc	4·1b

[a]Control: untreated; CS: *C. sake* at 5×10^7 colony-forming units mL^{-1}; FC: Fungicover at 50 g L^{-1}; FC25: Fungicover at 25 g L^{-1}; ×4: four applications from flowering to harvest; ×2: two applications in early season (80% flowering and pre-bunch closure).
[b]All values are means of four replicates. Mean values with the same letter are not significantly different ($P = 0{\cdot}05$) according to LSD Student's *t*-test.

berries from 10 bunches were also weighed and then immersed in 50 mL phosphate buffer. All sampled tissues were shaken for 20 min at 150 rpm on a rotary shaker and then sonicated for 10 min in an ultrasonic bath (JP Selecta S.L.). After serial dilutions, 100-μL aliquots were plated onto NYDA plates supplemented with streptomycin sulphate (0·5 g L^{-1}) for *C. sake* population estimates, or on Bengal rose agar (Biokar Diagnostics) plates amended with chloramphenicol (0·1 g L^{-1}) for estimates of *U. oudemansii* populations. Duplicate plates were incubated in the dark at 25°C, and colony counts measured after 48 h (for *C. sake*) and after 6 days (for *U. oudemansii*). For both BCAs, colonies were visually recognized based on their morphological characteristics. Data were collected as CFU mL^{-1} and expressed as CFU g^{-1}.

Microvinification of *C. sake*-treated grapes (2009 and 2010)

Grape bunches presenting similar ripeness and without bunch rot symptoms were visually selected at harvest and then transported to the winery for processing. In 2009, 10 kg grapes were collected from each treatment replicate plot, as fermentation was performed in small batches of 1 L. In 2010, 30 kg were also sampled from each of the four replicates of the evaluated treatments, conducting microvinifications in 20-L tanks. In both years, the grapes harvested from four replicate plots of the field experiment were redistributed into three replicate batches for the fermentation evaluation.

Grapes were crushed and pressed, sulphite added (40 mg L^{-1}) and the crushed juice allowed to settle overnight. After racking off the lees, the clear juice was inoculated with selected wine yeast (Lallemand EC1118). Fermentations were monitored by measuring density and quantity of residual sugar. Once the sugars were fermented, the wine was racked off the lees, cold-stabilized and then filtered.

Key wine quality parameters (ethanol, volatile acidity, reducing sugars, pH, titratable acidity and malic acid) were analysed following OIV methods (O.I.V., 2008). A descriptive sensory analysis was also performed by a trained panel (12 people) with samples treated with *C. sake* and Fungicover in 2010, following the standards of the International Organization for Standardization (ISO, 2008); wine samples were tasted in triplicate in tastes held on different days. Each tasting was conducted in individual tasting booths at room temperature (22°C). In each case, wines (50 mL) were served in coded, tulip-shaped wine glasses covered with plastic lids. Samples were presented in random order. Still mineral water was available for rinsing between wines.

Statistical analysis

Analysis of variance was performed using JMP®8 statistical discovery software (SAS Institute Inc.), for all data sets. Significant treatment differences were determined using LSD test ($P = 0{\cdot}05$). Orthogonal contrasts were used in the analysis of botrytis bunch rot incidence in the 2010 season to gain greater understanding of the overall treatment effects. CFU data for BCA population counts were log-transformed prior to ANOVA to stabilize the variance.

Results

2009 field studies

Botrytis bunch rot incidence at harvest was high (80·0%) in untreated plots, and average bunch rot severity was 8·2% (Table 1). All treatments significantly reduced ($P < 0{\cdot}05$) botrytis bunch rot incidence by 45% (CS low + FC) to 64% (CS + FC), and severity from 73% (AZ) to 90% (CS + FC) compared with the untreated control. When the low rate of *C. sake* (CS low + FC, 1×10^7 CFU mL^{-1}) was applied, botrytis bunch rot incidence increased and was significantly higher than with the standard (high) rate of *C. sake* (5×10^7 CFU mL^{-1}, CS + FC). There were no significant differences ($P < 0{\cdot}05$) in disease severity among applied treatments.

2010 field studies

Incidence and severity of botrytis bunch rot in the untreated control was higher in 2010 (89·5% and 21·7% respectively; Table 2) than in 2009. All treatments significantly reduced ($P < 0{\cdot}05$) botrytis bunch rot

incidence by 30% (FC25 × 2) to 67% (CS + FC × 4) and severity by 74% (FC × 4) to 89% (CS + FC × 4) compared to the untreated control. The most effective treatment was CS + FC × 4, which reduced botrytis bunch rot incidence from 89% (untreated control) to 30%. Botrytis bunch rot severity in treated plots was always lower than 5·6%, with no significant differences among treatments.

Overall, treatments with *C. sake* had significantly ($P < 0{\cdot}001$) less botrytis bunch rot incidence than treatments with Fungicover only (Table 3). Treatments including the high dose of Fungicover (50 g L^{-1}) presented significantly ($P < 0{\cdot}05$) lower incidence than those applied at the lower dose (25 g L^{-1}). Contrasts analysis also indicated that overall botrytis bunch rot control was significantly better ($P < 0{\cdot}05$) when four applications of *C. sake* and Fungicover were applied during the growing season, compared with two applications. None of the interactions among *C. sake* presence in treatments, Fungicover dose and number of applications were significant ($P < 0{\cdot}05$), as expressed in Table 3.

Meteorological data

Temperature and relative humidity data between each growth stage for both seasons are summarized in Table 4. The 2009 season was characterized by relatively constant conditions before véraison, with warm average temperatures and RH over 59% from late flowering to pre-bunch closure, followed by a very hot and dry period from véraison to before harvest. In 2010, cooler temperatures and higher RH were measured than in the previous year, except for the period from late flowering to pre-bunch closure, in which the average RH registered was the lowest of either season. From véraison to harvest, the 2010 season was consistently cooler and more humid than the 2009 season.

Population dynamics of biological control agents

The population dynamics of *C. sake* and *U. oudemansii* over time are summarized in Figures 1 and 2. CFU counts for each BCA treatment were high immediately after application, then declined over time. Populations of *C. sake* in the 2009 season (Fig. 1) were similar when comparing the different *C. sake* treatments, and ranged from 10^6 to 10^4 CFU g^{-1} sample when the concentration of the yeast in the spray suspension was 5×10^7 CFU mL^{-1}. When the lower concentration of *C. sake* was applied (CS low + FC), recovered populations were approximately one log unit lower at all sample times, compared with the application of the *C. sake* at the high rate. However, these differences in populations were significant ($P < 0{\cdot}05$) only at five sample times: 5% flowering, prior to 80% flowering, pre-bunch closure, 7 days before harvest and harvest (data not shown).

The *C. sake* populations followed the same pattern after véraison, except the (CS + FC)-AZ treatment that did not receive any additional *C. sake* treatment. Populations on berries decreased gradually in these samples, and at harvest had declined to the point where they could not be detected.

The population dynamics of *U. oudemansii*, BZ-(CS + FC) and BZ-AZ treatments are shown in Figure 1. The populations of *U. oudemansii* on grape tissues over time were similar in the BZ-AZ and BZ-(CS + FC) treatment samples. On green flowers, *U. oudemansii* populations ranged from 4·2 to 4·8 log (CFU g^{-1}) after spray application. Between 5% and 80% flowering, population counts declined by one log unit, whereas between the end of flowering and pre-bunch closure CFU g^{-1} declined ($P < 0{\cdot}05$) from 6 to 2 log.

No more BZ applications were applied after that time and *U. oudemansii* populations declined steadily to the point where they were not detected at harvest. The last

Table 3 Orthogonal contrasts testing treatment effects on botrytis bunch rot incidence in 2010 field experiment. Coefficients of equal value indicate pooled levels of the factor, coefficients of opposite sign indicate factor levels to be contrasted and zero indicates excluded factor levels

	Treatments[a]									
Effect test[b]	Control	CS + FC × 4	CS + FC × 2	CS + FC25 × 4	CS + FC25 × 2	FC × 4	FC × 2	FC25 × 4	FC25 × 2	*P*
C. sake	0	1	1	1	1	−1	−1	−1	−1	<0·0001
FC dose	0	1	1	−1	−1	1	1	−1	−1	0·016
*C. sake**FC dose	0	1	1	−1	−1	−1	−1	1	1	0·242
Number of applications	0	1	−1	1	−1	1	−1	1	−1	0·046
*C. sake**Number of applications	0	1	−1	1	−1	−1	1	−1	1	0·451
FC dose*Number of applications	0	1	−1	−1	1	1	−1	−1	1	0·242
*C. sake**FC dose*Number of applications	0	1	−1	−1	1	−1	1	1	−1	0·353

[a]Control: untreated; CS: *Candida sake* at 5×10^7 colony-forming units mL^{-1}; FC: Fungicover at 50 g L^{-1}; FC25: Fungicover at 25 g L^{-1}; ×4: four applications from flowering to harvest; ×2: two applications in early season (80% flowering and pre-bunch closure).

[b]*C. sake*: comparing treatments with or without *C. sake* CPA-1; FC dose: comparing treatments with Fungicover at 25 or 50 g L^{-1}; number of applications: comparing treatments with two or four applications; interactions among these effects are marked by *.

Table 4 Vine growth stages and meteorological data from a commercial organic vineyard in Catalonia in 2009 and 2010 seasons

	2009			2010		
Vine phenology	Dates	Average RH (%)[a]	Average temperature (°C)	Dates	Average RH (%)	Average temperature (°C)
5% flowering	8 June	54·5	22·4	12 June	64·7	16·3
80% flowering	15 June	59·3	22·5	21 June	49·7	24·2
Pre-bunch closure	2 July	63·8	22·9	12 July	67·7	22·7
Véraison	11 August	54·7	25·1	18 August	67·2	22·9
21 days before harvest	25 August	68·9	21·3	1 September	71·3	20·1
Harvest	14 September			15 September[b]		

[a]Average temperature and RH values are means of the daily mean values of each variable during the period between dates.
[b]Harvest date in the 2010 season was brought forward by the vineyard manager because of the high levels of botrytis bunch rot registered.

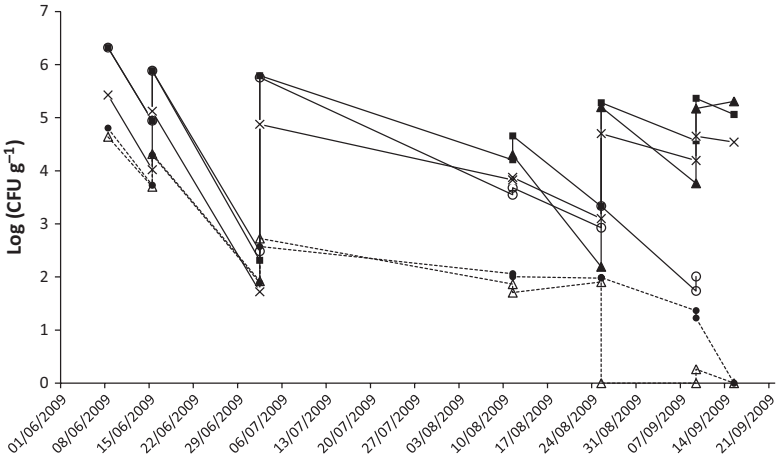


Figure 1 Population dynamics of two biological control agents *Candida sake* CPA-1 and *Ulocladium oudemansii*, applied to Macabeu wine grapes in a commercial organic vineyard in 2009. Early season applications were carried out between 5% flowering and pre-bunch closure, late-season applications were applied from véraison to harvest. Solid lines represent *C. sake* and dotted lines represent *U. oudemansii* colony-forming units (CFU). Treatments were: CS + FC (■): *C. sake* (CS) at 5 × 10^7 CFU mL^{-1}; CS low + FC (×): *C. sake* at 1 × 10^7 CFU mL^{-1}; BZ-AZ (●): three applications of *U. oudemansii* (BZ) at 2·5 × 10^6 CFU mL^{-1} early season and then three applications of chitosan (AZ) at 1·44 g L^{-1} (véraison to harvest); BZ-(CS + FC): three applications of *U. oudemansii* at 2·5 × 10^6 CFU mL^{-1} (Δ) (early season) and then three applications of *C. sake* at 5 × 10^7 CFU mL^{-1} (▲) (late season); (CS + FC)-AZ (○): three applications of *C. sake* at 5 × 10^7 CFU mL^{-1} (early season) and then three applications of chitosan at 1·44 g L^{-1} (late season). For each application, chitosan and *U. oudemansii* were applied with a generic wetting agent at 0·5 mL L^{-1}, chitosan treatments included 1·05 g $NaHCO_3$ L^{-1} and *C. sake* was applied with the additive Fungicover (FC) at 50 g L^{-1}. Flower or berry samples were taken after spraying and again just prior to the next spray application. CFU values are per gram of tissue sampled and were log-transformed. Values are the means of four replicates.

application was at pre-bunch closure and populations recovered at this stage were two log units lower than those after flowering treatments, ranging from 1·8 to 2·7 log (CFU g^{-1}) before véraison. After véraison populations decreased gradually in BZ-AZ-treated plots. In contrast, there was a substantial decline in the BZ-(CS + FC) treatment.

In 2010, the population dynamics of *C. sake* treatments were measured (Fig. 2). The populations of *C. sake* followed a similar pattern, which was predictable because the BCA concentration was the same for each treatment. No significant differences in *C. sake* populations were detected ($P < 0·05$) when Fungicover concentration was reduced from 50 to 25 g L^{-1} in any of the sampling dates (data not shown). After véraison, the *C. sake* population decreased gradually when only two applications of *C. sake* were made over the growing season, and were consistently over 10^2 CFU g^{-1}.

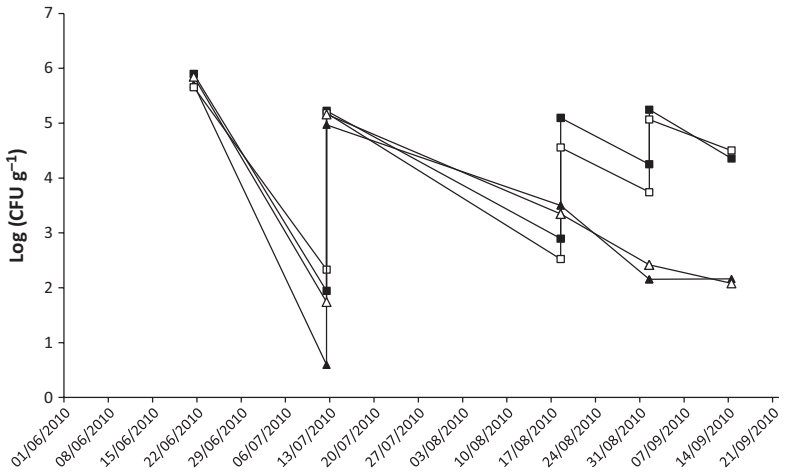


Figure 2 Population dynamics of *Candida sake* CPA-1 applied in 2010 field experiments to control botrytis bunch rot in Macabeu wine grapes. Treatments were *C. sake* (CS) at 5×10^7 colony-forming units (CFU) mL^{-1} with Fungicover at 50 g L^{-1} [CS + FC × 4 (■) and CS + FC × 2 (▲)], or Fungicover at 25 g L^{-1} [CS + FC25 × 4 (□) and CS + FC25 × 2 (Δ)]. Treatments labelled × 4 were applied four times from flowering to harvest; those labelled × 2 were applied at 80% flowering and pre-bunch closure. Flower or berry samples were taken after spraying and just before the next spray application. CFU values obtained per gram of sample tissue were log-transformed and values shown are means of four replicates.

Wine quality parameters

Results of final wine analysis are listed in Table 5. All wines fermented correctly, finishing the sugar, reaching the same alcohol amount and with normal volatile acidity considering the small size of the 2009 fermenters. In 2010 with 20 L fermentation, wines were also finished correctly with low residual sugar, similar alcohol content and low volatile acidity. In 2010, total acidity, pH and malic acid were also measured. Significant differences ($P < 0{\cdot}05$) were detected in the wine quality parameters evaluated (Table 5). Such differences may be produced by variability in grape ripeness. The results indicated that the vineyard treatments did not affect the normal fermentation of the juice and that the resulting wines showed normal analytical composition. No significant differences were detected among treatments in the fermentation dynamics at any sampling time (data not shown).

In 2010 the grapes were harvested before full maturity and presented higher acidity and lower alcohol levels than usual in wines elaborated in the area. Nevertheless, the wine tasting conducted by a trained panel showed no differences among the wines in colour, aroma and taste (data not shown).

Discussion

The urgent need for biologically based alternatives for control of *B. cinerea* in grapes has stimulated much research in the last two decades on a variety of natural products and BCAs. Because *C. sake* CPA-1 demonstrated effective control of botrytis bunch rot of grapes in conventional vineyards in 2005 and 2006 (Cañamás *et al.*, 2011), this study tested the efficacy of *C. sake* treatments in organic vineyards, evaluating different application timings as well as BCA and additive doses. Comparison with other registered products and integration of these strategies was also evaluated.

High botrytis bunch rot reductions achieved by full-season *C. sake* and Fungicover treatments in both seasons coincide with those reported by Cañamás *et al.* (2011) in 2006 (90% and 95% reductions in incidence and severity, respectively). The application of a low-rate *C. sake* treatment (CS low + FC) also significantly reduced both the incidence and severity of botrytis bunch rot, indicating that reduced rates of this BCA are a possible strategy in the future. However, the incidence of botrytis bunch rot was significantly higher in this treatment than with the full rate of *C. sake*, indicating that there may be some loss of field efficacy when a lower dose is applied, probably related to reported lower population counts in CS low + FC plots. Therefore, the standard rate of 5×10^7 CFU mL^{-1} is the preferred BCA concentration for effective and consistent control of botrytis bunch rot in grapes.

In the 2010 studies, once *C. sake* efficacy was confirmed to be similar to that of other commercialized products, the research focus was field evaluation of fewer *C. sake* applications per season and reduced rates of Fungicover. Botrytis bunch rot disease pressure in the vineyard was very high in 2010 compared with 2009, favoured by more suitable meteorological conditions for *B. cinerea* infection. Despite greater disease pressure, four applications at key phenological growth stages were sufficient for effective botrytis bunch rot control. Two applications of *C. sake* plus Fungicover before véraison were less effective than full-season treatments. Nonetheless, registered efficacy by treatments applied twice was also elevated, providing low-input alternatives for disease control, especially if climatic conditions after véraison

Table 5 Wine quality parameters after fermentation of Macabeu grapes using microvinification in 2009 and 2010

Treatment	Ethanol (% v/v)	Volatile acidity (g L^{-1})	Reducing sugars (g L^{-1})	pH	Titratable acidity (g L^{-1})	Malic acid (g L^{-1})
2009[a]						
Control	10·58a[c]	0·53a	0·43a	NA[d]	NA	NA
CS + FC	10·58a	0·40a	0·53a	NA	NA	NA
CS low + FC	10·79a	0·37a	0·47a	NA	NA	NA
AZ	10·56a	0·53a	0·23a	NA	NA	NA
BZ-AZ	10·22a	0·47a	0·30a	NA	NA	NA
BZ-(CS + FC)	10·20a	0·37a	0·20a	NA	NA	NA
(CS + FC)-AZ	10·68a	0·30a	0·30a	NA	NA	NA
FC	10·57a	0·30a	0·43a	NA	NA	NA
SED	0·590	0·081	0·243			
2010[b]						
Control	9·62a	0·20ab	0·26b	2·80c	8·13ab	2·70b
CS + FC × 4	9·39b	0·14c	0·26b	2·77d	7·90ab	2·50d
FC × 4	9·32b	0·21a	0·13b	2·88a	7·73b	2·73b
CS + FC25 × 4	9·35b	0·12d	0·86a	2·85b	7·03c	1·90d
FC25 × 4	8·69c	0·19b	0·06b	2·83b	8·23a	3·10a
FC + CS × 2	9·51ab	0·16c	0·23b	2·84b	7·86ab	2·63c
SED	0·061	0·005	0·115	0·004	0·142	0·019

[a]Control: untreated; CS + FC: (*Candida sake* at 5×10^7 colony-forming units (CFU) mL^{-1} + Fungicover at 50 g L^{-1}); CS low + FC (*C. sake* at 1×10^7 CFU mL^{-1} + Fungicover at 50 g L^{-1}); AZ: (chitosan at 1·44 g L^{-1} + wetting agent at 0·5 mL L^{-1} + $NaHCO_3$ at 1·05 g L^{-1}); BZ: (*Ulocladium oudemansii* at $2·5 \times 10^6$ CFU mL^{-1} + wetting agent at 0·5 mL L^{-1}); FC: (Fungicover at 50 g L^{-1}).

[b]Control: untreated CS: *C. sake* at 5×10^7 CFU mL^{-1}; FC: Fungicover at 50 g L^{-1}; FC25: Fungicover at 25 g L^{-1}; ×4: four applications from flowering to harvest; ×2: two applications in early season (80% flowering and pre-bunch closure).

[c]Mean values with the same letter are not significantly different ($P = 0·05$) according to LSD test.

[d]Not assessed.

are not highly conducive for *B. cinerea* development. Consistent botrytis bunch rot control under a wide range of conditions in 2006 (Cañamás *et al.*, 2011), 2009 and 2010 represents a significant new advance, because previous researchers have questioned whether BCAs can significantly reduce botrytis bunch rot infection when conditions are highly conducive to disease development (Metz *et al.*, 2002). Moreover, observed efficacy was comparable to other registered products in 2009, corroborating the potential of this BCA for commercial application.

When Fungicover was applied as a standalone product, botrytis bunch rot incidence and severity were significantly reduced. The precise mechanism of action of Fungicover against *B. cinerea* is poorly understood, but the manufacturers claim that infection is reduced by a physical barrier that this product forms on fruit surfaces after application (BioDúrcal, 2010). The effect of the BCA compared with treatments with Fungicover alone was not significant in 2009. However, the evaluation of *C. sake* and Fungicover treatments carried out in 2010 showed a highly significant BCA effect ($P < 0·001$). The effect of Fungicover on *C. sake* survival has been previously reported (Cañamás *et al.*, 2011). This additive also protects the yeast antagonist from exposure to external factors, while a certain nutritive effect has also been observed (C. Calvo-Garrido, N. Teixidó, unpublished data), which may favour the mode of action of *C. sake* based on nutrient competition. However, the interaction between *C. sake* and Fungicover dose was not significant and BCA survival was similar for both doses of the additive. Therefore, despite the high efficacy of treatments with *C. sake* plus a high dose of Fungicover, a synergistic effect is unlikely to occur.

Six applications of chitosan during the growing season resulted in botrytis bunch rot control equivalent to that with *C. sake*, and comparable to that reported in New Zealand vineyards over two growing seasons (Reglinski *et al.*, 2010). The treatment with *U. oudemansii* and then chitosan (BZ-AZ) also reduced botrytis bunch rot incidence and severity, achieving similar reduction levels to those reported by Reglinski *et al.* (2010) and other studies applying *U. oudemansii* alone or combined with synthetic fungicide programmes in New Zealand (Elmer *et al.*, 2005). The results reported here and in New Zealand are in contrast with those of Mehofer *et al.* (2009), who field-tested both BOTRY-Zen and ARMOUR-Zen for three seasons on Riesling grapes in Germany. These findings indicate a significant interaction between the performance of biologically based products, climatic regions and wine grape varieties, but this hypothesis requires further investigation. This research also represents the first vineyard study with *U. oudemansii* in a hot and dry climate, demonstrating its potential as an effective BCA under these conditions.

Three integrated programmes combining BCAs and natural products were also evaluated, namely BZ-AZ, BZ (CS + FC) and (CS + FC)-AZ, which resulted in sig-

nificant and effective reductions of botrytis bunch rot control, equivalent to reductions achieved with *C. sake* alone. Thus, integrated programmes did not significantly improve botrytis bunch rot control in the conditions tested. However, a combination of strategies with multiple modes of action has been recommended to ensure more consistent disease control and overcome fluctuations in external factors (Guetsky *et al.*, 2001; Elad & Stewart, 2004; Elmer & Reglinski, 2006).

Survival patterns for the two *C. sake* concentrations applied were similar and population numbers on flowers and the berry surface over time in 2009 and 2010 corresponded well with the findings reported by Cañamás *et al.* (2011). The combination of *C. sake* and Fungicover resulted in good field survival and persistence of this BCA on grape berries late in the growing season and occurred at a time when BCA persistence on the grape bunch may be variable (Holz & Volkmann, 2002). Populations of *C. sake* CPA-1 significantly declined across all treatments from 10^6 to 10^2 CFU g^{-1} host tissue between flowering and pre-bunch closure in both seasons, despite different meteorological conditions. During this period, many floral tissues abscise after the last flowering spray and there are also major physiological changes taking place as the pollinated flowers become berries. The volume of tissue on developing fruitlets increases, increasing the surface area of tissue not previously treated and thereby affecting the proportion of CFU recovered per gram. These two processes may explain the low population counts that were recovered prior to pre-bunch closure application.

Candida sake populations on berries after pre-bunch closure declined between each application and this pattern was repeated between the different *C. sake* treatments. The rate of decline was progressively lower in both seasons, providing some evidence for a gradual establishment of yeast populations on developing grape berries during the season, as originally hypothesized by Cañamás *et al.* (2011). It is also possible that survival was favoured by more moderate temperatures in the grape canopy in the final 21 days before harvest. Population declines between applications are dependent on external factors such as temperature, RH, UV radiation and rain exposure, while studies on the direct effects of these factors are currently being developed for a better understanding of *C. sake* population dynamics. Nonetheless, the survival pattern of *C. sake* CPA-1 was comparable to that of other antagonist yeasts that were field-applied: declining between spray applications, but, overall, remaining above 10^2 CFU g^{-1} (Schena *et al.*, 2000). Some yeast population studies reported more stable populations over time in postharvest conditions (Schena *et al.*, 1999; Zahavi *et al.*, 2000), but botrytis bunch rot control was lower.

On BZ-(CS + FC) treated berries, *C. sake* populations declined after the first application at véraison, and this coincided with the driest period of the whole season, with high temperatures that may have decreased early establishment by *C. sake*. During this same period *U. oudemansii* populations were relatively stable. After the 21-days-preharvest spray of *C. sake* and Fungicover, *C. sake* populations reached predictable numbers, while *U. oudemansii* CFU counts decreased, initially suggesting some incompatibility between the early season BCA on this tissue and *C. sake*. However, previous preliminary laboratory studies and results from an identical experiment carried out in a conventional vineyard in the same region (data not shown) did not show any incompatibility between *U. oudemansii* and *C. sake* CPA-1. This research is the first report that quantifies the survival of the *B. cinerea* antagonist *U. oudemansii* on green grapevine tissues. Results indicated that populations of *U. oudemansii* were relatively stable between flowering and pre-bunch closure, an important attribute for a BCA dependent upon necrotic tissues that at the time of application are green. *Ulocladium oudemansii* CFU counts recovered from green flowers were similar to those reported by Elmer & Kohl (1998) on lily leaves treated with the *B. cinerea* antagonist *U. atrum* 385 (2×10^6 CFU mL^{-1}). Low population counts on the grape berry surface indicated that this BCA found this ecological niche to be more hostile than the floral tissues. Nonetheless, the populations on the grape berries were relatively stable at 1×10^2 CFU mL^{-1} until 21 days before harvest.

Several fungi, particularly yeasts, can adversely affect grape fermentation and wine quality parameters. Consequently, microvinifications were carried out with treated grapes in both 2009 and 2010 to analyse possible effects of BCA treatments on fermentation. The progression of wine fermentation was similar among the different treatments and no relevant differences were detected in the wine quality parameters analysis in 2009 or 2010. Moreover, trained panel tasting did not reveal any fault in wines elaborated with *C. sake*-treated grapes in 2010.

All the biologically based strategies evaluated significantly reduced botrytis bunch rot incidence and severity, providing further evidence that alternatives to synthetic fungicides can achieve consistent, effective and practical control. *Candida sake* CPA-1, combined with Fungicover, is an attractive new BCA that is able to survive environmentally harsh conditions on the grape berry surface. Applications of this BCA resulted in high average efficacy for two seasons under a range of conditions in organic vineyards, indicating that this yeast-based BCA is a good candidate for future larger-scale field application with standard viticultural sprayers. Furthermore, wine quality was not adversely affected by *C. sake* applications and lower input treatments achieved promising results. These findings represent a significant advance towards commercialization of this BCA in the near future.

Acknowledgements

The authors are grateful to the University of Lleida for CCG's PhD grant and organic production research pro-

ject, the European Union (INTERREG REDBIO project) for their financial support and the Catalan Government (for a travel-assistance grant) and the Plant and Food Research Institute for travel assistance for PAGE.

References

BioDúrcal, 2010. Fungicover®. [http://www.biodurcal.com/media/Ficha FUNGICOVER.pdf]. Accessed 22 August 2012.

Cañamás TP, Viñas I, Torres R, Usall J, Solsona C, Teixidó N, 2011. Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. *Biological Control* **56**, 150–8.

El Ghaouth A, Wilson CL, Wisniewski M, 2003. Control of postharvest decay of apple fruit with *Candida saitoana* and induction of defense responses. *Phytopathology* **93**, 344–8.

Elad Y, Stewart A, 2004. Microbial control of *Botrytis* spp. In: Elad Y, Williamson B, Tudzynski P, Delen N, eds. Botrytis: *Biology, Pathology and Control*. Dordrecht, the Netherlands: Springer, 223–41.

Elmer PAG, Kohl J, 1998. The survival and saprophytic competitive ability of the *Botrytis* spp. antagonist *Ulocladium atrum* in lily canopies. *European Journal of Plant Pathology* **104**, 435–47.

Elmer PAG, Michailides TJ, 2004. Epidemiology of *Botrytis cinerea* in orchard and vine crops. In: Elad Y, Williamson B, Tudzynski P, Delen N, eds. Botrytis: *Biology, Pathology and Control*. Dordrecht, the Netherlands: Springer, 243–72.

Elmer PAG, Reglinski T, 2006. Biosuppression of *Botrytis cinerea* in grapes. *Plant Pathology* **55**, 155–77.

Elmer PAG, Hoyte SM, Vanneste JL, Reglinski T, Wood PN, Parry FJ, 2005. Biological control of fruit pathogens. *New Zealand Plant Protection* **58**, 47–54.

El-Neshawy SM, El-Morsy FM, 2003. Control of gray mold of grape by pre-harvest application of *Candida oleophila* and its combination with a low dosage of Euparen. *Acta Horticulturae* **600**, 95–102.

Fermaud M, Liminana JM, Froidefond G, Pieri P, 2001. Grape cluster microclimate and architecture affect severity of *Botrytis* rot of ripening berries. *Bulletin OILB/SROP* **24**, 7–9.

Fuster PMC, 2006. *Variedades de Vid: Registro de Variedades Comerciales*, 2nd edn. Madrid, Spain: Ministerio de Agricultura, Pesca y Alimentacion.

Guetsky R, Shtienberg D, Elad Y, Dinoor A, 2001. Combining biocontrol agents to reduce the variability of biological control. *Phytopathology* **91**, 621–7.

Hill G, Stellwaagkittler F, Huth G, Schlosser E, 1981. Resistance of grapes in different developmental stages to *Botrytis cinerea*. *Journal of Phytopathology* **102**, 328–38.

Holz G, Volkmann A, 2002. Colonisation of different positions in grape bunches by potential biocontrol organisms and subsequent occurrence of *Botrytis cinerea*. *Bulletin OILB/SROP* **25**, 9–12.

Holz G, Coertze S, Williamson B, 2004. *The Ecology of* Botrytis *on Plant Surfaces*. Dordrecht, the Netherlands: Springer.

ISO, 2008. *ISO 5492:2008*. Sensory Analysis – Vocabulary. Geneva, Switzerland: International Organization for Standardization.

Jackson RS, 2008. *Wine Science: Principles and Applications*. London, UK: Academic Press.

Jacometti MA, Wratten SD, Walter M, 2010. Review: alternatives to synthetic fungicides for *Botrytis cinerea* management in vineyards. *Australian Journal of Grape and Wine Research* **16**, 154–72.

Komárek M, Čadková E, Chrastný V, Bordas F, Bollinger JC, 2010. Contamination of vineyard soils with fungicides: a review of environmental and toxicological aspects. *Environment International* **36**, 138–51.

Leroch M, Kretschmer M, Hahn M, 2011. Fungicide resistance phenotypes of *Botrytis cinerea* isolates from commercial vineyards in south west Germany. *Journal of Phytopathology* **159**, 63–5.

MARM, 2010. Estadísticas 2010 Agricultura Ecológica – España. [http://www.marm.es/es/alimentacion/temas/la-agricultura-ecologica/INFORME_NACIONAL_2010_(4)_tcm7-171341.pdf]. Accessed 15 February 2012.

Mehofer M, Hanak K, Diwald M, Regner F, 2009. Investigations into botrytis control in organic-biological viticulture. *Mitteilungen Klosterneuburg* **59**, 189–98.

Metz C, Oerke EC, Dehne HW, 2002. Biological control of grey mould (*Botrytis cinerea*) with the antagonist *Ulocladium atrum*. *Mededelingen Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen Universiteit Gent* **67**, 353–9.

Nally MC, Pesce VM, Maturano YP *et al.*, 2012. Biocontrol of *Botrytis cinerea* in table grapes by non-pathogenic indigenous *Saccharomyces cerevisiae* yeasts isolated from viticultural environments in Argentina. *Postharvest Biology and Technology* **64**, 40–8.

O.I.V., 2008. *Compendium of International Methods of Wine and Must Analysis*. Paris, France: O.I.V.

Parry F, Elmer P, Wood P *et al.*, 2011. Developing new biologically-based products for control of botrytis bunch rot. Part 2: developing a new biologically-based product for late-season botrytis control: the BCA-L1 story. *Wine and Viticulture Journal* **26**, 73–8.

Reglinski T, Elmer PAG, Taylor JT, Wood PN, Hoyte SM, 2010. Inhibition of *Botrytis cinerea* growth and suppression of botrytis bunch rot in grapes using chitosan. *Plant Pathology* **59**, 882–90.

Romanazzi G, Gabler FM, Margosan D, Mackey BE, Smilanick JL, 2009. Effect of chitosan dissolved in different acids on its ability to control postharvest gray mold of table grape. *Phytopathology* **99**, 1028–36.

Schena L, Ippolito A, Zahavi T, Cohen L, Nigro F, Droby S, 1999. Genetic diversity and biocontrol activity of *Aureobasidium pullulans* isolates against postharvest rots. *Postharvest Biology and Technology* **17**, 189–99.

Schena L, Ippolito A, Zahavi T, Cohen L, Droby S, 2000. Molecular approaches to assist the screening and monitoring of postharvest biocontrol yeasts. *European Journal of Plant Pathology* **106**, 681–91.

Scherm B, Ortu G, Muzzu A, Budroni M, Arras G, Migheli Q, 2003. Biocontrol activity of antagonistic yeasts against *Penicillium expansum* on apple. *Journal of Plant Pathology* **85**, 205–13.

Torres R, Teixidó N, Viñas I *et al.*, 2006. Efficacy of *Candida sake* CPA-1 formulation in controlling *Penicillium expansum* decay on pome fruit from different Mediterranean regions. *Journal of Food Protection* **69**, 2703–11.

Wurms K, Chee A, Elmer P, Agnew R, Wood P, Chee AA, 2011. Developing new biologically-based products for control of botrytis bunch rot. Part 1: developing a new natural product for mid-season botrytis control – NP2 moves closer to the market. *Wine and Viticulture Journal* **26**, 64–72.

Xu WT, Huang KL, Guo F *et al.*, 2007. Postharvest grapefruit seed extract and chitosan treatments of table grapes to control *Botrytis cinerea*. *Postharvest Biology and Technology* **46**, 86–94.

Zahavi T, Cohen L, Weiss B *et al.*, 2000. Biological control of *Botrytis*, *Aspergillus* and *Rhizopus* rots on table and wine grapes in Israel. *Postharvest Biology and Technology* **20**, 115–24.

CAPÍTULO 2

Suppression of *Botrytis cinerea* on necrotic grapevine tissues by early-season applications of natural products and biological control agents

C. Calvo-Garrido, I. Viñas, P.A.G. Elmer, J. Usall and N. Teixidó

Pest Management Science **(en prensa), DOI 10.1002/ps.3587**

Research Article

Received: 6 February 2013 Revised: 16 May 2013 Accepted article published: 6 June 2013 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.3587

Suppression of *Botrytis cinerea* on necrotic grapevine tissues by early-season applications of natural products and biological control agents

Carlos Calvo-Garrido,[a] Inmaculada Viñas,[a] Philip AG Elmer,[b] Josep Usall[c] and Neus Teixidó[c]*

Abstract

BACKGROUND: Necrotic tissues within grape (*Vitis vinifera*) bunches represent an important source of *Botrytis cinerea* inoculum for *Botrytis* bunch rot (BBR) at harvest in vineyards. This research quantified the incidence of *B. cinerea* on necrotic floral and fruit tissues and the efficacy of biologically based treatments for suppression of *B. cinerea* secondary inoculum within developing bunches.

RESULTS: At veraison (2009 and 2010), samples of aborted flowers, aborted fruits and calyptras were collected, and the incidence and sporulation of *B. cinerea* were determined. Aborted fruits presented significantly higher incidence in untreated samples. Early-season applications of *Candida sake* plus Fungicover®, Fungicover alone or *Ulocladium oudemansii* significantly reduced *B. cinerea* incidence on aborted flowers and calyptras by 46–85%. Chitosan treatment significantly reduced *B. cinerea* incidence on calyptras. None of the treatments reduced *B. cinerea* incidence on aborted fruits. Treatments significantly reduced sporulation severity by 48% or more.

CONCLUSIONS: Treatments were effective at reducing *B. cinerea* secondary inoculum on necrotic tissues, in spite of the variable control on aborted fruits. This is the first report to quantify *B. cinerea* on several tissues of bunch trash and to describe the effective suppression of saprophytic *B. cinerea* inoculum by biologically based treatments.

Keywords: biological control; *Candida sake* CPA-1; chitosan; floral debris; *Ulocladium oudemansii* HRU3; *Vitis vinifera*

1 INTRODUCTION

Several infection pathways have been described for *Botrytis cinerea*, the causal agent of *Botrytis* bunch rot (BBR) of wine grapes, and these were summarised by Elmer and Michailides.[1] It is generally acknowledged that early-season infection by *B. cinerea* of senescent and necrotic floral grape tissues during flowering and pre-bunch closure determine bunch rot at harvest,[2–4] while late-season infections of intact mature berries are associated with both conidial and mycelial inoculum sources from infection of the style, stamens, calyptra, aborted flowers and aborted berries.[1] Other necrotic tissues have been identified as potential inoculum sources within developing bunches and include leaf fragments or tendrils,[5,6] or the ring of necrotic tissue at the pedicel–receptacle complex ('cap scar'),[2,7] whereas tissues trapped within the ripening bunch have been considered important sources of *B. cinerea* for late-season development of BBR, especially when bunches are compact.[8] In addition, other epidemiological studies have also shown the high relative importance of treatments early in the season, which interfere with the pathways described above, for effective BBR control at harvest.[2,9]

This knowledge of the importance of early-season infections has provided opportunities for more targeted chemical control.[10,11] However, the application of synthetic fungicides in vineyards worldwide has become less popular owing to the ease with which *B. cinerea* populations quickly adapt to new fungicide chemistry[12–14] and reports of harmful environmental and human health risks associated with some synthetic fungicides.[15] Constraints associated with fungicide use and the increasing number of organic growers highlight the need for alternative or complementary control strategies against BBR.

In organic viticulture, synthetic botryticides are not permitted, and the number of alternative products available for BBR control is reduced to the application of salts, essential oils, compost and plant extracts.[16,17] Moreover, results are sometimes variable at

* *Correspondence to: Neus Teixidó, IRTA, XaRTA-Postharvest, 191 Rovira Roure, 25198 Lleida, Catalonia, Spain. E-mail: neus.teixido@irta.cat*

a *Food Technology Department, Lleida University, XaRTA-Postharvest, Agrotecnio Center, Lleida, Catalonia, Spain*

b *The New Zealand Institute for Plant and Food Research Limited, Ruakura Research Centre, Hamilton, New Zealand*

c *IRTA, XaRTA-Postharvest, Lleida, Catalonia, Spain*

harvest, and specific early-season treatments are very limited. In response, several research groups worldwide have investigated alternative, organically acceptable treatments such as those based upon biological control agents (BCAs), natural products (NPs) and biologically based elicitors of host defence for *Botrytis* control in viticulture. The mode of action of these strategies is usually preventive, and the curative effect is generally reduced. Thus, applications early in the season are highly recommended in order to achieve a significant control of secondary inoculum and hence prevent BBR epidemic outbreak, especially when dealing with alternative treatments.

In New Zealand, an isolate of *Ulocladium oudemansii* (HRU3) consistently reduced BBR in grapes, while saprophytic colonisation of senescent and necrotic calyptras and aborted fruitlets early in the growing season was identified as its primary mode of action.[16,18,19] This isolate was successfully commercialised and is the active ingredient in BOTRY-Zen®, a BCA product that is approved for early-season *Botrytis* suppression in organic and conventional viticulture in New Zealand. Suppression of BBR development was when this BCA was integrated with mid- and late-season commercialised natural products, including chitosan.[19,20]

Chitosan is a natural carbohydrate polymer extracted from crustaceans, and several studies report the efficacy of different chitosan fractions against several phytopathogenic fungi,[21,22] including *B. cinerea* on grapes.[23–25]

The CPA-1 strain of *Candida sake* was isolated, optimised and formulated by the Postharvest Pathology research group at IRTA, Lleida. When it was field applied with the additive Fungicover® to wine grapes in conventional and organic vineyards in Catalonia, Spain, BBR at harvest was significantly reduced.[24,26] Fungicover is a natural product consisting of an emulsion of fatty acids and polysaccharides in aqueous–alcoholic solution, and it is principally used by researchers as a coating and protective agent for *C. sake* field applications able to improve BCA survival.[26]

The biologically based products described above have shown *B. cinerea* control under a variety of conditions. However, the ability of *U. oudemansii* to suppress *B. cinerea* on necrotic tissues in hot, dry winegrowing regions has not been reported, and there are no published data on the effect of chitosan or *C. sake* applications on *B. cinerea* development in necrotic bunch debris.

The objectives of this study were (a) to quantify the relative incidence of *B. cinerea* infections on different necrotic tissues within bunches of organic wine grapes 'Macabeu' and (b) to determine the efficacy of early-season applications of three different biologically based treatments in terms of their ability to reduce *B. cinerea* inoculum.

2 MATERIALS AND METHODS

2.1 Vineyard field trials

Field trials were established in two commercial vineyards (identified here as OLV and CTD) and were carried out over two growing seasons. The vineyards were 10 km apart and located in a traditional winemaking area (designation of origin Costers del Segre, Lleida, Catalonia, Spain). This region typically experiences cold winters and hot, dry summers (mean annual precipitation 428.4 mm, mean average temperature 13.9 °C).[27] The winemaking variety selected was 'Macabeu', a white variety characterised by a large, compact bunch structure that is highly susceptible to BBR. The vines in OLV and CTD were trained to a goblet system and were planted 2.5 m apart between the rows and 2 m apart within each row.

The OLV vineyard was certified organic by the Catalan Committee for Organic Agriculture Production (CCPAE) and was sprayed to control plant diseases other than BBR. In 2009, the vineyard received three applications of 99% sulphur (w/w) prior to flowering, one more application of 99% sulphur (w/w) plus 98% silicon (w/w) at pre-bunch closure and finally one application of *Bacillus thuringiensis* var. Kurstaki at veraison. In 2010, treatments included one application of 99% sulphur (w/w) prior to flowering, two applications of 60% sulphur (w/w) plus 4% copper oxychloride (w/w) at pre-bunch closure, one application of 38% copper oxychloride (w/w) at pre-bunch closure and two applications of *B. thuringiensis* var. Kurstaki at veraison. The CTD vineyard was conventionally managed and received one 80% sulphur spray (w/w) at pre-bunch closure, but no antibotrytic fungicide applications. All products in both fields were applied at the doses and application rates recommended by the product manufacturers.

Two investigations were carried out in 2009 and 2010. In the first study, a range of necrotic tissues were sampled from untreated grape bunches in order to quantify the incidence and sporulation potential of *B. cinerea* infections in necrotic bunch trash tissues of the white wine variety Macabeu. In the second study, necrotic tissues were sampled from developing grape bunches at veraison in order to determine the effect of biologically based treatments on *B. cinerea* development on treated necrotic bunch trash debris. In the first study, OLV and CTD vineyards were sampled in 2009, whereas in 2010 only the OLV vineyard was used. The second study was carried out in the OLV vineyard in 2009 and 2010.

2.2 Part 1: Source of *B. cinerea* on necrotic tissues in developing bunches of Macabeu

At veraison, samples of necrotic tissues that were trapped within the developing bunches were taken from four bunches per replicate. Bunches were randomly selected from the sample vines, and in the laboratory each bunch was divided into four sections (top left, top right, middle and bottom of the bunch). Three different samples of necrotic tissues were collected with sterile tweezers from each bunch section, consisting of one aborted flower, one calyptra and one aborted fruit. Aborted fruits were considered to be different from aborted flowers when the swollen ovary of the transforming flower presented a diameter greater than 2 mm. Trays, gloves and tweezers were surface sterilised with ethanol prior to sectioning of each bunch and removal of necrotic samples.

The four samples of each tissue type from each bunch were placed on a sterile petri dish with Whatman No. 1 (85 mm diameter) filter paper that had been moistened with 1.25 mL of sterile distilled water to create a high-humidity chamber for *B. cinerea* development. The four high-humidity chambers corresponding to four bunches constituted one replicate. In total there were 16 aborted flowers, calyptras and aborted fruit per replicate. The petri dishes were then incubated at 20 °C in the dark for 10 days prior to assessment.

The sampling protocol described in this section was the same for both experiments. In the first study, samples were taken from untreated plots only, while, in the second study, samples were taken both from plots that had received biologically based treatments and from untreated plots.

2.3 Part 2: Effect of biologically based treatments on *B. cinerea* colonisation of necrotic floral tissues

2.3.1 Microbial antagonists and natural products

The BCA *C. sake* CPA-1 was originally isolated from apples by the University of Lleida IRTA centre and was deposited at the Coleccion Española de Cultivos Tipo (CECT-10817) at the University of Valencia, Burjassot, Spain. Stock cultures were stored on nutrient yeast dextrose agar (NYDA) medium (nutrient broth, 8 g L^{-1}; dextrose 10 g L^{-1}; agar 15 g L^{-1}) at 4 °C. When required, *C. sake* CPA-1 was subcultured onto NYDA plates at 25 °C. Then, subcultured cells suspended on potassium phosphate buffer (KH_2PO_4 0.2 M, 70 mL; K_2HPO_4 0.2 M, 30 mL; deionised water 300 mL) were added as inoculum starter to 5 L of molasses-based (MB) medium (cane molasses 40 g L^{-1}; urea 1.2 g L^{-1}; water activity $a_w = 0.996$), with adjustment of the initial concentration to 1×10^6 CFU mL^{-1}. Cell pellets were obtained by centrifugation at 6831 × *g* for 10 min at 10 °C after 40 h of liquid fermentation at 25 °C, 400 rpm agitation speed and 150 L h^{-1} aeration level. Resuspended pellets were then formulated in an isotonic solution, with adjustment of the water potential with trehalose as described previously [28] Prior to field application, the BCA additive Fungicover (Biodúrcal S.L., Granada, Spain) was added to the *C. sake* suspension (50 g L^{-1}) and then mixed with the aid of a handheld paint mixer to ensure thorough mixing. Fungicover was included because this additive has been shown to aid field survival of *C. sake*.[26]

The BCA *U. oudemansii* was obtained as a dry, water-dispersible granule (BOTRY-Zen®, AI *U. oudemansii* 2.5×10^8 CFU L^{-1}; Botry-Zen 2010 Ltd, Dunedin, New Zealand). Chitosan was sourced from ARMOUR-Zen® as a liquid concentrate (AI chitosan 1.44 g L^{-1}; Botry-Zen 2010 Ltd, Dunedin, New Zealand). Because the water-dispersible granule and liquid concentrate formulations did not contain wetting agents, final solutions for field trials were prepared in water containing 0.5 mL L^{-1} of the wetting agent Mojante Inagra (alquil poliglicol 20% w/v; Sipcam Inagra S.A., Valencia, Spain) to improve tissue wetting. For the field application of chitosan treatment, 1.05 g L^{-1} of sodium bicarbonate was added to the final solution to adjust to pH = 7, as it can improve *B. cinerea* suppression by chitosan (Reglinski T, private communication).

2.3.2 Experimental design

In 2009, six early-season treatments were evaluated for their ability to reduce *B. cinerea* infection of necrotic bunch tissues. The treatments and timing of applications during the growing season are summarised in Table 1. In 2010, treatments consisted of two applications of *C. sake* CPA-1 and Fungicover at 80% flowering and pre-bunch closure (Table 1).

The 2009 and 2010 experiments, including biologically based treatments, were carried out in the OLV field, with four replicate plots containing seven vines for each treatment laid out in a randomised block design. All treatments in both seasons were applied with a motorised backpack sprayer (WJR2225 model; Honda Motor Company Ltd, Germany) at 1.5×10^6 Pa pressure and with a 1 mm nozzle focusing on grape bunches to the point of run-off.

After treatment applications, necrotic tissue samples from treated and untreated plots were collected and processed at veraison, as previously described for the first study evaluating inoculum sources.

Table 1. Summary of treatments applied early in the growing season for *B. cinerea* suppression in an organic vineyard cv. Macabeu in 2009 and 2010

Treatment[a]	1–5% flowering	80% flowering	Pre-bunch closure
2009			
Control	—	—	—
Chitosan	Chitosan	Chitosan	Chitosan
U. oudemansii	*U. oudemansii*	*U. oudemansii*	*U. oudemansii*
CS + FC	CS + FC	CS + FC	CS + FC
CS low + FC	CS low + FC	CS low + FC	CS low + FC
FC	FC	FC	FC
2010			
Control	—	—	—
CS + FC	—	CS + FC	CS + FC
FC	—	FC	FC

[a] Control: untreated; Chitosan: chitosan 1.44 g L^{-1} + wetting agent 0.5 mL L^{-1} + 1.05 g L^{-1} of $NaHCO_3$; *U. oudemansii*: *U. oudemansii* 2.5×10^6 CFU mL^{-1} + wetting agent 0.5 mL L^{-1}; CS: *C. sake* 5×10^7 CFU mL^{-1}; CS low: *C. sake* 1×10^7 CFU mL^{-1}; FC: Fungicover 50 g L^{-1}. Treatments consisted of three applications. Applications were carried out over four replicates of each treatment in a randomised block design.

2.4 Colonisation assessment of *B. cinerea* and *U. oudemansii* in necrotic bunch trash tissues

In both the first and the second studies in 2009 and 2010, each necrotic tissue sample was visually assessed using a stereomicroscope after 10 days of incubation in high-humidity chambers. The sporulation severity of *B. cinerea* was measured using a sporulation index with a 0–5 scale, where 0 = no visible conidiophores, 1 = 1–5, 2 = 6–10, 3 = 11–20, 4 = 21–40 and 5 = 40–100 conidiophores. In samples treated with *U. oudemansii* in 2009, the area of necrotic tissues covered in sporulating *U. oudemansii* was determined using a 0–100% scale, where no *U. oudemansii* visible = 0%, trace quantities visible = 1–5%, low–moderate colonisation = 6–25%, moderate colonisation = 26–50%, moderate–high colonisation = 51–75% and high colonisation = 76–100%. The mean percentage value of *U. oudemansii* colonisation was calculated for each class in the scale (0, 3, 15.5, 38, 63 and 88% respectively) and then multiplied by the number of samples in each class eventually to obtain the average percentage coverage per replicate tissue type.

To calculate the mean values of *B. cinerea* incidence, *B. cinerea* sporulation severity, *U. oudemansii* incidence and percentage tissue area colonised by *U. oudemansii*, use was made of data from visual assessment of the 16 necrotic samples of each tissue type per replicate.

2.5 *Botrytis* bunch rot assessment

BBR at harvest in the untreated plots used in the bunch trash studies was measured in the 2009 and 2010 seasons at the field sites by assessing 50 bunches per replicate plot (25 bunches from each side of the plot). BBR incidence (number of bunches with *B. cinerea*) was expressed as a percentage, and BBR severity was visually estimated as the percentage of the bunch infected.

2.6 Meteorological data

Hourly measurements of temperature (*T*), relative humidity (RH), rainfall (RN) and leaf wetness (LW) were collected using a weather

station (Decagon Services Inc., Pullman, WA) placed beside one of the experimental plots in the two seasons of the study.

2.7 Statistical analysis

Analysis of variance was performed using JMP8 (SAS Institute Inc., Cary, NC) for all datasets. Significant treatment differences were determined using Student's LSD *t*-test ($P = 0.05$) for the effect of biologically based treatments on *B. cinerea*. Tukey's test ($P = 0.05$) was used to separate necrotic tissue means for significant differences. Sporulation index data of *B. cinerea* samples were transformed [$\sqrt{(x + 0.5)}$] prior to ANOVA to improve homogeneity of variances.

3 RESULTS

3.1 Source of *B. cinerea* on necrotic tissues in developing bunches of Macabeu

B. cinerea was detected on all necrotic tissue types sampled from untreated plots at both sites (CTD and OLV in 2009) and in both years (OLV in 2009 and 2010) (Fig. 1). No significant interactions ($P < 0.05$) were detected between years, field sites or *B. cinerea* incidence (data not shown). Therefore, incidence data in the different tissue types for 2009 and 2010 and the two field sites could be pooled for the statistical analysis.

Overall, the incidence of *B. cinerea* in aborted fruits of Macabeu (38%) was significantly higher ($P < 0.05$) than the incidence in aborted flowers or calyptras (18% and 24% respectively) (Fig. 1).

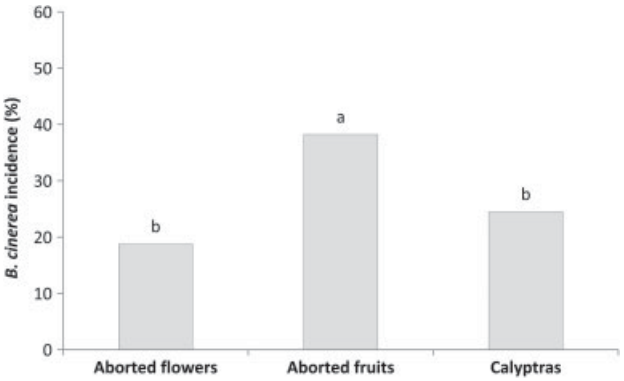


Figure 1. Incidence of *B. cinerea* natural infections in necrotic tissues sampled from within immature grape bunches from untreated plots. Data are average of two years and two sites (OLV field in 2009 and 2010; CTD field in 2009). Values are the means of four replicate plots. Mean values with the same letter are not significantly different ($P = 0.05$) according to Tukey's test.

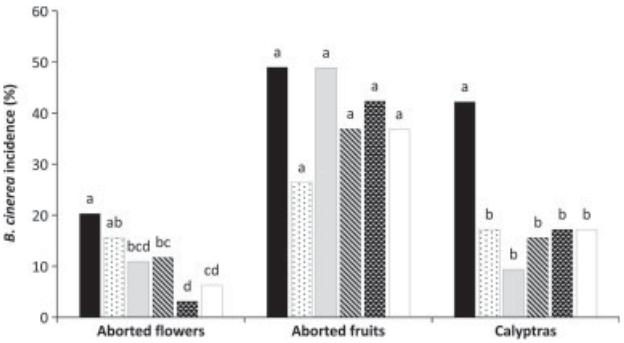


Figure 2. Effect of early-season biologically based treatments on *B. cinerea* incidence in necrotic tissues from grape bunches of Macabeu sampled at 2009 veraison in the OLV organic vineyard. Control (■): untreated; Chitosan (□): chitosan 1.44 g L^{-1} + wetting agent 0.5 mL L^{-1} + 1.05 g L^{-1} of $NaHCO_3$; *U. oudemansii* (▤): *U. oudemansii* 2.5 × 10^6 CFU mL^{-1} + wetting agent 0.5 mL L^{-1}; CS + FC (▧): *C. sake* 5 × 10^7 CFU mL^{-1} + Fungicover 50 g L^{-1}; CS low + FC (▩): *C. sake* 1 × 10^7 CFU mL^{-1}; FC (▥): Fungicover 50 g L^{-1}. Treatments were applied to vineyard plots at 1–5% flowering, 80% flowering and pre-bunch closure. Values are the mean of four replicate plots. Mean values with the same letter are not significantly different ($P = 0.05$) according to LSD Student's t-test.

3.2 Reduction of *B. cinerea* incidence and sporulation in necrotic tissues by early-season biologically based treatments – 2009 field studies

The incidence of *B. cinerea* on untreated necrotic tissues in 2009 compared with tissues that had been field sprayed with biologically based treatments is summarised in Fig. 2. In the untreated plots the incidence of *B. cinerea* ranged from 20% in aborted flowers to 42 and 48% in calyptras and aborted fruits respectively. All treatments significantly reduced ($P < 0.05$) *B. cinerea* incidence in the necrotic calyptras, ranging from 59% (Chitosan, CS low + FC, FC) to 77% (*U. oudemansii*). All treatments, with the exception of chitosan, significantly reduced *B. cinerea* incidence in the aborted flowers ($P < 0.05$) by 46% (*U. oudemansii*) to 84% (CS low + FC) compared with the untreated control. Overall, there were no significant differences ($P < 0.05$) in *B. cinerea* incidence among the effective treatments for any tissue type, with the exception of CS + FC and CS low + FC in the aborted flowers. Three applications of the biologically based treatments did not significantly reduce *B. cinerea* incidence ($P < 0.05$) in the aborted fruits.

The mean number of *B. cinerea* conidiophores across all tissue types was expressed using a sporulation index and was 0.97 from samples taken from the untreated plots (Fig. 3). This value equates to an average of 1–5 conidiophores per tissue sample. All biologically based treatments significantly reduced the *B. cinerea* sporulation index value ($P < 0.05$) by 50% or more compared with untreated samples (Fig. 3). There were no significant differences between the treatments ($P < 0.05$), and the sporulation index ranged from 0.43 (Chitosan) to 0.51 (CS + FC).

3.3 Colonisation of necrotic tissues by *U. oudemansii*

Sampled tissues from the *U. oudemansii*-treated blocks in 2009 were further examined for *U. oudemansii* incidence and the extent of tissue colonisation by this BCA. The incidence of *U. oudemansii* was significantly higher ($P < 0.05$) on aborted flowers (66%) compared with aborted fruits or the calyptras (26 and 41% respectively), as shown in Fig. 4.

A breakdown of the extent of *U. oudemansii* colonisation on the different tissue types indicated some interesting patterns (Fig. 4). Samples presenting trace quantities represented 60, 77 and 86% of the aborted flowers, aborted fruits and calyptras with *U. oudemansii* present. Samples with low–moderate colonisation were reduced in aborted fruits and calyptras (respectively 5 and 3% of samples) but more abundant in aborted flowers (22% of samples). Moderate colonisation was only observed in aborted flowers (3 and 2% of samples respectively), and only 0.8% of aborted flowers showed high colonisation. This difference in the ability to colonise the different tissue types was also evidenced by the average percentage of colonised tissue (solid diamond in each bar). In aborted flowers, the average percentage of colonised tissue was 6.3% and was significantly higher ($P < 0.05$) compared with the aborted fruits (1.5%) and calyptras (2.4%).

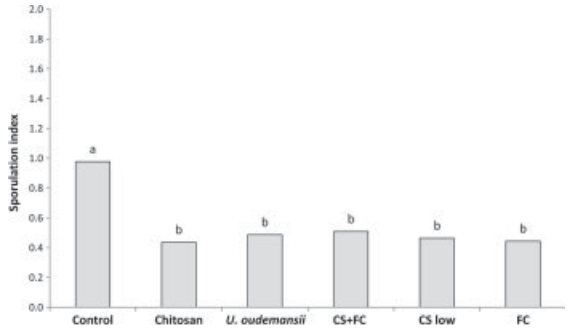


Figure 3. Effect of early-season biologically based treatments on *B. cinerea* sporulation severity in necrotic trash sampled from grape bunches of Macabeu in 2009 in the OLV organic vineyard. Control: untreated; Chitosan: Chitosan 1.44 g L^{-1} + wetting agent 0.5 mL L^{-1} + 1.05 g L^{-1} of $NaHCO_3$; *U. oudemansii*: *U. oudemansii* 2.5 × 10^6 CFU mL^{-1} + wetting agent 0.5 mL L^{-1}; CS + FC: *C. sake* 5 × 10^7 CFU mL^{-1} + Fungicover 50 g L^{-1}; CS low + FC: *C. sake* 1 × 10^7 CFU mL^{-1}; FC: Fungicover 50 g L^{-1}. Represented values are the means of four replicate plots, except for the U. oudemansii and CS + FC treatments which are the means of eight replicate plots. Sporulation was measured visually using a sporulation index (0–5), where 0 = no visible conidiophores on the tissue, 1 = 1–5, 2 = 6–10, 3 = 11–20, 4 = 21–40 and 5 = 40–100. Mean values with the same letter are not significantly different ($P = 0.05$) according to LSD Student's t-test

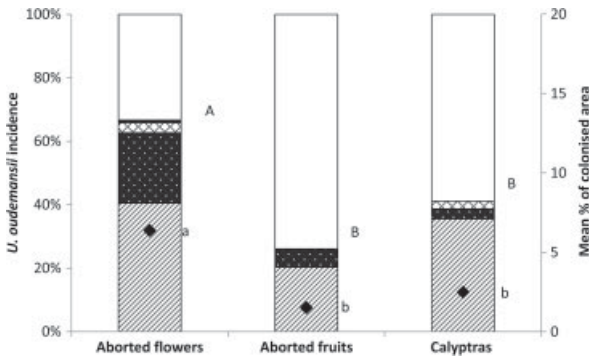


Figure 4. Incidence (bars) and percentage of necrotic tissue colonised by *Ulocladium oudemansii*-like conidiophores (solid diamond shape) from tissue samples removed from grape bunches cv. Macabeu at veraison in 2009. Treated plots received three applications of *U. oudemansii* between 5% flowering and veraison, applied at 2.5 × 10^6 CFU mL^{-1} + 0.5 mL L^{-1} of Mojante Inagra wetting agent. No *U. oudemansii* visible (□) = 0%; trace quantities visible (▨) = 1–5%; low–moderate colonisation (▩) = 6–25%; moderate colonisation (▩) = 26–50%; moderate–high colonisation (■) = 51–75%; high colonisation (■) = 76–100%. Values are means of eight replicates. Mean values with the same upper-case or lower-case letter are not significantly different ($P = 0.05$) according to Tukey's test.

3.4 Reduction of *B. cinerea* incidence in necrotic tissues by early-season biologically based treatments – 2010 field studies

In the 2010 experiment, the average incidence of *B. cinerea* across all tissue types in the untreated control plots was 14% (Fig. 5). The CS + FC and FC treatments significantly reduced *B. cinerea* incidence in necrotic tissues ($P < 0.05$) by 57 and 65% respectively. There were no significant differences between the CS + FC and FC treatments.

3.5 Meterological data, *B. cinerea* in early season and bunch rot at harvest

The temperature, relative humidity, rainfall and leaf wetness data between the early and late part of the growing season and the *B. cinerea* incidence in bunch trash at veraison and BBR at harvest in

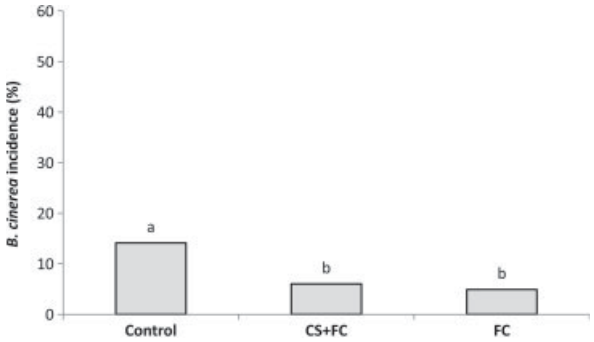


Figure 5. Effect of *Candida sake* and Fungicover on *B. cinerea* incidence on necrotic tissues sampled at 2010 veraison in the OLV organic vineyard. Treatments were applied at 50% flowering and pre-bunch closure. Control: untreated; CS + FC: *C. sake* 5 × 10^7 CFU mL^{-1} + Fungicover 50 g L^{-1}; FC: Fungicover 50 g L^{-1}. Mean values with the same letter are not significantly different ($P = 0.05$) according to LSD Student's t-test.

2009 and 2010 are summarised in Table 2. Rainfall in the early part of the growing season (flowering to veraison) was very similar in both seasons, but there were eight rain episodes in 2009 (Fig. 6) compared with four in 2010. Longer leaf wetness duration most likely accounted for the significantly higher incidence of *B. cinerea* on necrotic floral tissues in 2009 (30–37%) compared with 14% in 2010. However, the high *B. cinerea* inoculum detected in the trash at veraison did not correspond to high BBR severity at harvest, and the significantly lower leaf wetness duration in the late part of the growing season in 2009 (840 min) may have accounted for the lack of BBR development late season.

In contrast, in 2010 there was a low incidence of *B. cinerea* on necrotic bunch trash sampled at veraison, but a substantially longer leaf wetness duration in the late part of the growing season, which resulted in significantly greater BBR severity at harvest in 2010 compared with 2009. Leaf wetness was especially regular in the last 15 days before harvest of the 2010 season, with 13 periods in 2 weeks. Late season in 2009 was hotter and dryer, especially during the first 2 weeks after veraison, and no leaf wetness periods were detected until 14 days before harvest. These findings indicate that late-season wetness duration could be a key driver for BBR development late season compared with *B. cinerea* inoculum potential in the bunch.

4 DISCUSSION

This research represents the first investigation specifically aimed at quantifying the incidence of *B. cinerea* in a range of necrotic tissues trapped within developing grape bunches in a conventional and in an organic vineyard over two growing seasons. It is also the first report on the effect of biologically based treatments on *B. cinerea* saprophytic inoculum sources.

Results showed that a significantly higher overall incidence of *B. cinerea* infection of the bunch trash corresponded to more conducive conditions in the early part of the growing season in 2009 compared with 2010. This was probably related to abundant periods of leaf wetness, as mean temperature, RH and rainfall were similar in both years, while leaf wetness and *B. cinerea* incidence on necrotic tissues were higher in 2009. Under the inland Mediterranean conditions in which the study was carried out, RH is relatively low during the early season, and most of the periods of leaf wetness were directly associated with rain episodes. This suggests that the number of rain episodes, rather than accumulative

Table 2. Incidence of *B. cinerea* on bunch trash, *Botrytis* bunch rot incidence and severity at harvest and key meteorological data of the growing season in 2009 and 2010. Incidence and severity values are from untreated plots

Year	*Botrytis* incidence on bunch trash (%)[a]		*Botrytis* bunch rot incidence (%)	*Botrytis* bunch rot severity (%)	Early season (beginning of flowering to veraison)				Late season (veraison to harvest)			
					RH[b] (%)	*T* (°C)	Rainfall (mm)	Leaf wetness (min)	RH (%)	*T* (°C)	Rainfall (mm)	Leaf wetness (min)
2009	OLV CTD	37.1 a 30.2 a	80.0 a	8.2 b	61.8	22.8	25.0	4020	63.4	22.7	3.0	840
2010	OLV	14.1 b	89.5 a	21.7 a	61.9	22.1	21.7	1324	69.9	21.6	8.5	5252

[a] Values of the same variable linked by the same letter are not significantly different ($P = 0.05$) according to Tukey's test.
[b] RH: relative humidity; *T*: temperature; values are the means of daily mean *T* or RH.

rainfall, could be an important factor in BBR disease development. Few hours are needed by *B. cinerea* to infect grape flowers with the required temperature and RH,[29] and thus isolated rain events could provide favourable conditions for a sufficient time to infect green tissues or necrotic tissues. Moreover, wetness periods inside the bunch last longer, especially in a compact cluster cultivar such as Macabeu,[30] also providing long periods of wetness for saprophytic colonisation. Meteorological conditions also influenced disease development on berries resulting from necrotic tissues from veraison to harvest. In 2009, a high inoculum level at veraison did not correspond to higher bunch rot incidence and severity, while meteorological conditions late in the season favoured disease epidemics in 2010 in spite of a lower inoculum level. An unclear correlation between inoculum level in the bunch and grey mould at harvest was also observed in a 4 year study evaluating 56 field sites in France,[31] suggesting that climate is the more important variation factor. Wolf *et al*. also found a variable response of bunch rot to floral debris removal,[32] stating that other factors were interacting. Further studies are needed for a more precise understanding of the effect of bunch microclimate and meteorological variables on inoculum development inside the grape bunch.

In the present study, aborted fruits presented significantly more *B. cinerea* incidence, representing a significant source of potential inoculum. In other studies, infected aborted fruits were an important source of *B. cinerea* inoculum in the Hunter Valley in Australia,[33] and profuse sporulation on aborted fruits was also reported by Seyb,[6] who identified a significant relationship between the presence of aborted berries within bunches and berry infection at harvest, but not for other trash types, in the dry winegrowing region of Marlborough, New Zealand. However, the importance of aborted fruits for final BBR at harvest is likely to be reduced, as the frequency of aborted fruits in the bunch is generally lower than that of aborted flowers or calyptras. In the present experiments, although there was no exhaustive quantification of tissue types, the 12 aborted fruits per treatment × replicate were difficult to obtain from developing bunches, whereas aborted flowers and calyptras were very abundant. In accordance with these results, calyptras have been identified as the most abundant necrotic tissue inside the bunches in Australian vineyards, and,[34] in addition, the relevance of aborted fruits is also dependent upon the studied cultivar, vine management and several biotic and abiotic factors.[35,36]

In the 2009 studies, three treatment applications before veraison were effective at reducing *B. cinerea* inoculum potential in the calyptras and aborted flowers. In contrast, the reduction in *B. cinerea* on aborted fruitlets was much more variable, and none of the biologically based treatments significantly reduced *B. cinerea* on this inoculum source at veraison in this variety. Nevertheless, as the importance of aborted fruits for BBR is variable, as discussed above, the results suggest that biologically based treatments provide consistent inoculum control early in the growing season.

All treatments were also effective in reducing the *B. cinerea* sporulation index by approximately 50% compared with the untreated control. When samples presenting *B. cinerea* incidence were analysed separately (data not shown), no significant differences in sporulation index were detected, suggesting that sporulation control may be a consequence of incidence reduction rather than a specific effect of treatments on the sporulation process of the pathogen on infected tissues.

The evidence presented in this study further confirms that *U. oudemansii* is an effective antagonist of *B. cinerea* even when applied in comparatively hot and dry Mediterranean vineyard conditions. The findings are similar to those reported on the wine grape variety Sauvignon blanc in cool–temperate viticulture conditions in New Zealand vineyards, where incidence reductions were similar to those of the fungicide treatment.[18] In the present study, *B. cinerea* incidence reduction by *U. oudemansii* applications at the same three key points in the early season was lower (41% compared with 71%), but the incidence of *B. cinerea* in the untreated blocks was higher (37.1% compared with 28.7%). The cited study did not evaluate efficacy for each tissue separately. The ability of *U. oudemansii* to suppress *B. cinerea* is also supported by the significant incidence reduction achieved in calyptras and aborted flowers, although colonisation was categorised as trace or low–moderate for most of the samples with *U. oudemansii*.

The chitosan treatment was only effective in controlling *B. cinerea* incidence in calyptras. Reported modes of action of chitosan against *B. cinerea* range from host defence activation on green tissues[37] to direct antifungal activity of the product solution or the biofilm formed on grape berries.[23,38] After the flower parts senesce and aborted flowers become necrotic, direct antifungal activity is the only likely primary mode of action against *B. cinerea*.

No significant reduction was achieved by CS + FC compared with FC alone in 2009 or 2010 samples, indicating low efficacy of *C. sake* on this substrate. This result reveals interesting information on the mode of action of the *C. sake* and Fungicover combination for reducing grey mould at harvest. *C. sake* is effective at protecting green tissues when combined with FC, while FC by itself has some efficacy against *B. cinerea* infections as well.[24] In addition, FC is able significantly to reduce inoculum sources in the bunch. Possible modes of action of Fungicover include establishment of

a physical barrier,[39] which may prevent infection of senescent and necrotic floral tissues. Fungicover efficacy by itself and the protective effect observed on the yeast antagonist have identified this product as the preferred additive for *C. sake* application.

Although biologically based treatments effectively reduced *B. cinerea* inoculum, the contribution of *B. cinerea* control in necrotic tissues by treatments to the overall reduction in BBR at harvest is difficult to quantify, as it is dependent on meteorological conditions late in the season. Calvo-Garrido *et al*. evaluated the efficacy of the early-season treatments described in this work combined with late-season applications of chitosan or CS + FC.[24] All treatments in that study significantly reduced bunch rot incidence and severity in 2009 and 2010. Nonetheless, only the CS + FC and FC treatments, which consisted of two applications before veraison in 2010, can be examined to evaluate efficacy against bunch rot of the early-season treatments described in this study. Both reduced overall severity, by 85% (CS + FC) and 78% (FC), compared with the control.[24] The reduction rates were very high under favourable conditions for BBR development, confirming the efficacy at harvest of these early-season treatments, although reductions are the consequence of the control on necrotic tissues and other infection pathways not evaluated here, such as latent infections. In spite of the elevated efficacy of these treatments, the control achieved by alternative strategies may be affected by fluctuations in meteorological conditions. Therefore, spray timing may be modified depending on weather conditions during the season. For example, early in the season, preventive applications are desirable prior to rain episodes, whereas applications may be reduced to one at flowering if dry conditions are forecast before veraison. In contrast, inoculum reduction early in the season should be complemented with applications of other post-veraison strategies if meteorological conditions are conducive to infection.

Overall, the present results provide information for a better understanding of *B. cinerea* epidemics with the given cultivar and climatic characteristics, which can be extrapolated to similar warm and dry regions. The number of rain episodes was identified as an interesting indicator of *B. cinerea* development in necrotic tissues before veraison. In addition, aborted fruits showed higher *B. cinerea* incidence and represented the inoculum source most difficult to control by the tested treatments, although the influence of this source for BBR at harvest is variable owing to its low frequency in bunches. Alternative treatments to synthetic fungicides effectively controlled mycelial growth and sporulation of *B. cinerea* on bunch trash under different meteorological conditions. Thus, even if the contribution of early-season treatments to BBR reduction at harvest is dependent on meteorological conditions, the study highlights the potential of these BCAs and NPs as alternative strategies to control BBR of grapes.

ACKNOWLEDGEMENTS

The authors would like to thank the University of Lleida for Carlos Calvo-Garrido's PhD grant and organic production research project, the European Union (INTERREG REDBIO project) for their financial support and the Catalan Government for Dr Philip Elmer's travel assistance grant. Thanks also to Plant and Food Research for assistance with living expenses while at IRTA.

REFERENCES

1 Elmer PAG and Michailides TJ, Epidemiology of *Botrytis cinerea* in orchard and vine crops, in *Botrytis: Biology, Pathology and Control*, ed. by Elad Y, Williamson B, Tudzynski P and Delen N. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 243–272 (2004).
2 Keller M, Viret O and Cole FM, *Botrytis cinerea* infection in grape flowers: defense reaction, latency, and disease expression. *Phytopathology* **93**(3):316–322 (2003).
3 Viret O, Keller M, Jaudzems VG and Cole FM, *Botrytis cinerea* infection of grape flowers: light and electron microscopical studies of infection sites. *Phytopathology* **94**(8):850–857 (2004).
4 Nair NG, Guilbaud-Oulton S, Barchia I and Emmett R, Significance of carry over inoculum, flower infection and latency on the incidence of *Botrytis cinerea* in berries of grapevines at harvest in New South Wales. *Aust J Exp Agric* **35**(8):1177–1180 (1995).
5 Mundy DC, Agnew RH and Wood PN, Grape tendrils as an inoculum source of *Botrytis cinerea* in vineyards – a review. *NZ Plant Prot* **65**:218–227 (2012).
6 Seyb A, *Botrytis cinerea* inoculum sources in the vineyard system. Lincoln University, Christchurch (2004).
7 Holz G, Gütschow M, Coertze S and Calitz FJ, Occurrence of *Botrytis cinerea* and subsequent disease expression at different positions on leaves and bunches of grape. *Plant Dis* **87**(4):351–358 (2003).
8 Hed B and Travis JW, Effects of grape cluster compactness and debris retention on *Botrytis* bunch rot development. *Phytopathology* **95**(6):S169–S169 (2005).
9 Petit AN, Vaillant-Gaveau N, Walker AS, Leroux P, Baillieul F, Panon ML *et al.*, Determinants of fenhexamid effectiveness against grey mould on grapevine: respective role of spray timing, fungicide resistance and plant defences. *Crop Prot* **29**(10):1162–1167 (2010).
10 Shtienberg D, Rational management of *Botrytis*-incited diseases: integration of control measures and use of warning systems, in *Botrytis: Biology, Pathology and Control*, ed. by Elad Y, Williamson B, Tudzynski P and Delen N. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 335–347 (2004).
11 Fermaud M, Roudet J, Sauris P, Druelle L, Blanc G and Lagouarde P, Implementation of a low-input fungicide strategy in relation to the pattern of grey mold development. XV Int Botrytis Symp, Cadiz, Spain (2010).
12 Leroch M, Kretschmer M and Hahn M, Fungicide resistance phenotypes of *Botrytis cinerea* isolates from commercial vineyards in south west Germany. *J Phytopathol* **159**(1):63–65 (2011).
13 Latorre BA and Torres R, Prevalence of isolates of *Botrytis cinerea* resistant to multiple fungicides in Chilean vineyards. *Crop Prot* **40**:49–52 (2012).
14 Yan H, Yan J, Wang Z, Li Y, Jin G and Li X, Multiple fungicide resistance of *Botrytis cinerea* from grapevine to three fungicides. *J Fruit Sci* **29**(4):625–629 (2012).
15 Komárek M, Čadková E, Chrastný V, Bordas F and Bollinger JC, Contamination of vineyard soils with fungicides: a review of environmental and toxicological aspects. *Environ Int* **36**(1):138–151 (2010).
16 Elmer PAG and Reglinski T, Biosuppression of *Botrytis cinerea* in grapes. *Plant Pathol* **55**:155–177 (2006).
17 Jacometti MA, Wratten SD and Walter M, Review: Alternatives to synthetic fungicides for *Botrytis cinerea* management in vineyards. *Aust J Grape Wine Res* **16**(1):154–172 (2010).
18 Elmer PAG, Reglinski T, Wurms K and Wood PN, Enabling technologies: the practical integration of biological and natural products to disease control, in *Future Changes in Crop Protection: Repositioning NZ's Primary Industries*, ed. by Butcher MR, Walker JTS and Zydenbos SM. The New Zealand Plant Protection Society, Napier, New Zealand, pp. 109–116 (2007).
19 Parry F, Elmer P, Wood P, Agnew R, Saunders J, Wurms K *et al*., Developing new biologically-based products for control of botrytis bunch rot. Part 2: Developing a new biologically-based product for late-season botrytis control: the BCA-L1 story. *Wine Vitic J* **26**(5):73–78 (2011).
20 Wurms K, Chee A, Elmer P, Agnew R, Wood P and Chee AA, Developing new biologically-based products for control of botrytis bunch rot. Part 1: Developing a new natural product for mid-season botrytis control – NP2 moves closer to the market. *Wine Vitic J* **26**(5):64–72 (2011).
21 Badawy MEI and Rabea EI, Characterization and antimicrobial activity of water-soluble *N*-(4-carboxybutyroyl) chitosans against some plant pathogenic bacteria and fungi. *Carbohydr Polym* **87**(1):250–256 (2012).

22 Zhang H, Li R and Liu W, Effects of chitin and its derivative chitosan on postharvest decay of fruits: a review. *Int J Mol Sci* **12**(2):917–934 (2011).
23 Reglinski T, Elmer PAG, Taylor JT, Wood PN and Hoyte SM, Inhibition of *Botrytis cinerea* growth and suppression of botrytis bunch rot in grapes using chitosan. *Plant Pathol* **59**(5):882–890 (2010).
24 Calvo-Garrido C, Elmer PAG, Viñas I, Usall J, Bartra E and Teixidó N, Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1. *Plant Pathol* **62**:510–519 (2013).
25 Romanazzi G, Lichter A, Gabler FM and Smilanick JL, Recent advances on the use of natural and safe alternatives to conventional methods to control postharvest gray mold of table grapes. *Postharvest Biol Technol* **63**(1):141–147(2012).
26 Cañamás TP, Viñas I, Torres R, Usall J, Solsona C and Teixidó N, Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. *Biol Control* **56**(2):150–158 (2011).
27 *Climatologia. L'Urgell 1971–2000*. [Online]. Servei Meteorologic de Catalunya. Available: http://www20.gencat.cat/docs/meteocat/Continguts/Climatologia/climatologies_comarcals/documents/Urgell.pdf [4 February 2013].
28 Abadias M, Usall J, Teixidó N and Viñas I, Liquid formulation of the postharvest biocontrol agent *Candida sake* CPA-1 in isotonic solutions. *Phytopathology* **93**(4):436–442 (2003).
29 Nair NG and Allen RN, Infection of grape flowers and berries by *Botrytis cinerea* as a function of time and temperature. *Mycol Res* **97**:1012–1014 (1993).
30 Fermaud M, Liminana JM, Froidefond G and Pieri P, Grape cluster microclimate and architecture affect severity of *Botrytis* rot of ripening berries. *Bull OILB/SROP* **24**(7):7–9 (2001).
31 Coulon T, Epidemiological study of gray rot: no connection between early onset and final infection levels of *Botrytis cinerea* in french vineyards. *Prog Agricol Viticol* **120**(9):205–210 (2003).
32 Wolf TK, Baudoin A and Martinez-Ochoa N, Effect of floral debris removal from fruit clusters on botrytis bunch rot of Chardonnay grapes. *Vitis* **36**(1):27–33 (1997).
33 Nair NG and Parker FE, Midseason bunch rot of grapes – an unusual disease phenomenon in the Hunter Valley, Australia. *Plant Pathol* **34**(2):302–305 (1985).
34 Nair NG and Hill GK, Bunch rot of grapes caused by *Botrytis cinerea*, in *Plant Diseases of International Importance, Vol. III, Diseases of Fruit Crops*, ed. by Kumar J, Chaube HS, Singh US and Mukhopadhyay AN. Prentice Hall, Englewood Cliffs, NJ, pp. 147–169 (1992).
35 Caspari HW, Lang A and Alspach P, Effects of girdling and leaf removal on fruit set and vegetative growth in grape. *Am J Enol Vitic* **49**(4):359–366 (1998).
36 Hidalgo L, *Tratado de Viticultura General*. Ediciones Mundi-Prensa, Barcelona, Spain (1993).
37 Meng XH, Li BQ, Liu J and Tian SP, Physiological responses and quality attributes of table grape fruit to chitosan preharvest spray and postharvest coating during storage. *Food Chem* **106**:501–508 (2008).
38 Romanazzi G, Gabler FM, Margosan D, Mackey BE and Smilanick JL, Effect of chitosan dissolved in different acids on its ability to control postharvest gray mold of table grape. *Phytopathology* **99**(9):1028–1036 (2009).
39 *Ficha Técnica Fungicover*. [Online]. BioDúrcal. Available: http://www.biodurcal.com/media/Ficha%20FUNGICOVER.pdf [4 February 2013].

CAPÍTULO 3

Candida sake CPA-1 and other biologically based products as potential control strategies to reduce sour rot of grapes

C. Calvo-Garrido, I. Viñas, P.A.G. Elmer, J. Usall and N. Teixidó

Letters in Applied Microbiology **(2013), 57, 356-361**

Letters in
Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Candida sake CPA-1 and other biologically based products as potential control strategies to reduce sour rot of grapes

C. Calvo-Garrido[1], I. Viñas[1], P. Elmer[2], J. Usall[3] and N. Teixidó[3]

1 Food Technology Department, Lleida University, XaRTA-Postharvest, Agrotecnio Center, Lleida, Catalonia, Spain
2 The New Zealand Institute for Plant & Food Research Limited, Ruakura Research Centre, Hamilton, New Zealand
3 IRTA, XaRTA-Postharvest, Lleida, Catalonia, Spain

Significance and Impact of the Study: Studies on sour rot of grapes are scarce in literature, and this is the first work specifically evaluating sour rot in Spanish vineyards. Sour rot control in field conditions through applications of antagonistic micro-organisms is reported for first time in this study, showing elevated severity reductions (40–67% compared with control). As there are no options available for sour rot control in vineyards, results point *Candida sake* CPA-1 as an interesting control strategy against grape bunch rots.

Keywords
antagonist, biocontrol, grey mould, *Vitis vinifera*, yeast.

Correspondence
Neus Teixidó, IRTA, XaRTA-Postharvest ,191 Rovira Roure, 25198-Lleida, Catalonia, Spain.
E-mail: neus.teixido@irta.cat

2013/0564: received 22 March 2013, revised 14 June 2013 and accepted 14 June 2013

doi:10.1111/lam.12121

Abstract

Sour rot of grapes is becoming increasingly important disease in many winegrowing regions, while consistent chemical or biological control has not been reported. Authors evaluated relative incidence and severity of sour rot in untreated grapevines and the effect of different biologically based treatments on sour rot at harvest. Applications of *Candida sake* CPA-1 plus Fungicover®, *Ulocladium oudemansii* and chitosan were carried out in an organic vineyard in Lleida area, Spain, during the 2009 and 2010 growing seasons. At harvest, incidence and severity of sour rot were assessed. Significantly higher incidence and severity of sour rot were observed in untreated plots in 2009, when meteorological conditions after veraison were warmer. All treatments including *C. sake* CPA-1 significantly reduced ($P < 0{\cdot}05$) severity of sour rot in both seasons, ranging from 40 to 67% compared with the untreated control. Incidence of sour rot was not significantly reduced by any treatment. This study helps to characterize development of sour rot in the dry Mediterranean climate conditions of the experiment, whereas also represents the first report of biological control of sour rot. Treatments with the tested biologically based products are a promising strategy to control sour rot.

Introduction

Sour rot is a disease of grapes that has lately received interest by researchers due to its increasing economical importance in several grape production regions of the world, (Nigro *et al.* 2006; Oriolani *et al.* 2009; Huber *et al.* 2011; Wei *et al.* 2011). It is becoming especially frequent compared with the *Botrytis* bunch rot (*Botrytis cinerea*; BBR) in regions presenting hot summer season conditions. However, relationship between both diseases and factors determining their relative prevalence has been poorly studied, although an increase in non-*Botrytis* rots is foreseeable with global warming (Steel and Greer 2008). Disease aetiology is still not completely defined and is controversial, although most authors consider yeasts and bacteria as the main causing agents of rot (Guerzoni and Marchetti 1987; Gravot *et al.* 2001; Oriolani *et al.* 2009; Huber *et al.* 2011; Wei *et al.* 2011; Barata *et al.* 2012a). Common disease symptoms are browning and dislodgement of the berry tissue, detachment of berries from pedicel and leaking of juice with a notable acetic acid smell (Marchetti *et al.* 1984; Gravot *et al.* 2001). Another characteristic fact associated with sour rot of grapes is the presence of vinegar

flies (*Drosophila* spp.) around affected berries, which represent a main disease vector (Fermaud *et al.* 2002) that plays a crucial role in the epidemic dissemination in the vineyard, as recently observed by (Barata *et al.* 2012b).

Despite increasing importance of grape sour rot, there is a lack of control strategies. There are not synthetic fungicides available for growers, and chemical control is generally considered to be ineffective (Gravot *et al.* 2001; Nigro *et al.* 2006), although there are some reports of disease reduction (Rooney-Latham *et al.* 2007). In contrast, other strategies not based upon fungicide programmes have shown significant reductions. For example, mechanical and manual leaf removal (Stapleton and Grant 1992; Sivilotti *et al.* 2011), applications of salts (Nigro *et al.* 2006) and natural substances such as Gibberelines (Spring and Viret 2009). Studies on biological control of sour rot with antagonistic micro-organisms are scarce and, to our knowledge, significant reductions in *in vivo* experiments are only reported in one published work, in which a mixture of antagonist yeasts and natural products reduced sour rot up to 50% (Ligorio *et al.* 2007). However, there are no reports of reductions by field applications of biological control agents (BCAs) in vineyards, while the need for effective treatments against sour rot is urgent, encouraging research in new control options.

In this study, the objective was to evaluate the effect of different biological control agents and natural products, which had previously shown efficacy against BBR, on sour rot incidence and severity at harvest. Additionally, we characterized the meteorological conditions associated with sour rot incidence and severity in untreated grapevines at harvest during two growing seasons.

Results and Discussion

Effect of biologically based products applications on sour rot

Figure 1a summarizes the results of sour rot severity across treatments in 2009. Significant differences ($P < 0{\cdot}05$) were observed between the untreated control and the five treatments including applications of biological control agents, namely BZ-AZ, CS low + FC, (CS + FC)-AZ, BZ-(CS + FC) and CS + FC. Significant severity reductions ranged from 38% (BZ-AZ) to 52% (CS + FC) compared with control. Differences observed among these five treatments were not significant ($P < 0{\cdot}05$). All treatments applying *C. sake* and/or Fungicover significantly reduced ($P < 0{\cdot}05$) severity of sour rot in the 2010 season (Fig. 1b), with the exception of FC ×4 treatment. Reductions ranged from 47% (CS + FC25 ×2) to 70% (FC ×2) compared with untreated control.

Field efficacy rates achieved are similar to reductions reported by other BCA treatments in controlled conditions (Ligorio *et al.* 2007; Dimakopoulou *et al.* 2008). However, limited comparisons with other studies can be carried out, as there is a lack of published research on sour rot control by biologically based treatments.

The BCA effect was clearer in 2009 with higher sour rot disease pressure, when all the treatments including *C. sake* were significantly more effective than the FC treatment. However, the mechanism by which *C. sake* plus Fungicover treatments are able to reduce sour rot severity remains unknown. As nutrient competition is *C. sake* mode of action against other fungal pathogens, its application could be also reducing available niches for phylloplane micro-organisms like the sour rot pathogenic yeasts, although further studies on *C. sake* should be carried out to confirm efficacy and understand causes of variability and their mode of action.

In the 2009 season, incidence of sour rot was not reduced by any treatment and there were no significant differences ($P < 0{\cdot}05$) between the untreated control and the treatments. In the 2010 season, incidence in most of the treated plots was lower than in the control and the CS + FC ×2 treatment presented the lowest incidence. However, differences were not significant ($P < 0{\cdot}05$) as variability of data was high, as represented by standard error bars (Fig. 1b). The lack of efficacy reducing sour rot incidence may be very influenced by other external factors, such as disease spread by *Drosophila* flies, which are a main disease vector and are not likely to be affected by BCA treatments. In addition, two interesting trends were also observed regarding sour rot incidence in 2010: (i) treatments applying *C. sake* CPA-1 generally presented lower incidence than the analogous treatments applying Fungicover alone, suggesting a BCA effect; and (ii) treatments consisting of four applications presented higher sour rot incidence than treatments consisting of two applications, which could respond to a negative effect of FC applications late in the growing season. In addition to these trends, six applications in 2009 and four applications in 2010 of FC alone at the higher rate did not reduce sour rot severity, but treatments with the reduced rate (25 g l^{-1}) or applying only two times (×2) significantly reduced severity in 2010. To fully understand the unclear role of FC and other factors implicated in the efficacy of studied strategies, other interesting effects could be investigated, such as FC effect on yeasts and *Drosophila* populations, the importance of wetness periods at high temperatures after spray applications and the interactions among *B cinerea*, sour rot yeasts, *C. sake* and FC.

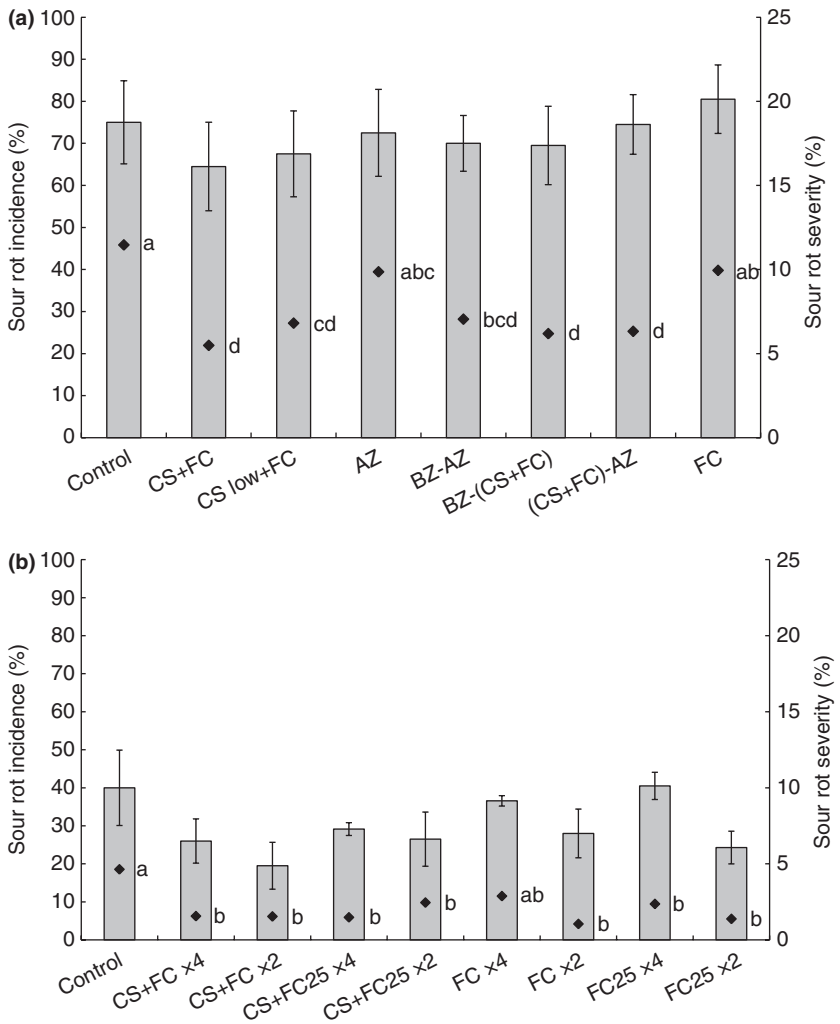


Figure 1 Incidence (bars) and severity (diamonds) of sour rot in Macabeu wine grapes at harvest in (a) 2009 and (b) 2010 growing seasons. Treatments in 2009 were: Control: untreated, CS + FC: six applications of *Candida sake* 5×10^7 CFU ml^{-1} + Fungicover 50 g l^{-1}; CS low + FC: six applications of *C. sake* 1×10^7 CFU ml^{-1} + Fungicover 50 g l^{-1}; AZ: six applications of chitosan 1·44 g l^{-1}; BZ-AZ: three applications of *Ulocladium oudemansii* $2{\cdot}5 \times 10^6$ CFU ml^{-1} in early season and then three applications of chitosan 1·44 g l^{-1} (veraison to harvest); BZ-(CS + FC): three applications of *U. oudemansii* $2{\cdot}5 \times 10^6$ CFU ml^{-1} (early season) and then three applications of *C. sake* 5×10^7 CFU ml^{-1} (late season); (CS + FC)-AZ: three applications of *C. sake* 5×10^7 CFU ml^{-1} (early season) and then three applications of chitosan 1·44 g l^{-1} (late season); FC: six applications of Fungicover 50 g l^{-1}. Treatments in 2010 were: Control: untreated; CS + FC: *C. sake* 5×10^7 CFU ml^{-1} + Fungicover 50 g l^{-1}; CS + FC25: *C. sake* 5×10^7 CFU ml^{-1} + Fungicover 25 g l^{-1} FC: Fungicover 50 g l^{-1}; FC25: Fungicover 25 g l^{-1}. Treatments were applied four times (×4) from flowering to harvest, or two times (×2) at 80% flowering and prebunch closure. Values are means of four replicates. Mean severity values linked by the same letter are not significantly different ($P = 0{\cdot}05$) according to LSD Student's *t*-test, whereas error bars represent standard error of nonsignificantly different incidence values.

Sour rot in untreated vines and meteorological data

Meteorological data sets collected between veraison and harvest in 2009 and 2010 are summarized in Table 1. The late part of the growing season (from veraison onwards) in 2009 was dryer and warmer than in 2010. Mean temperature and mean max temperature were higher in 2009 than in the same period during 2010. These variations in

Table 1 Summary of the meteorological data after veraison date in 2009 and 2010 growing seasons

	T (°C)	T_{max} (°C)	HR (%)	Hours $T > 30$°C	Days $T_{max} > 30$ °C
2009	22·7	29·5	63·4	88	17
2010	21·6	28·3	69·9	49	8

the meteorological conditions corresponded with changes in bunch rot at harvest.

Overall severity of sour rot in the control plots of the assay was higher ($P < 0{\cdot}05$) in 2009 compared with 2010, decreasing from 11·4 to 4·6% between both growing season (Fig. 1a,b). Sour rot incidence at harvest in the untreated plots significantly decreased from 75·0 to 40·0% comparing the 2009 and 2010 seasons, respectively. Thus, the reduced sour rot epidemic observed in 2010 was characterized by the significant decrease ($P < 0{\cdot}05$) of both incidence and severity compared with the 2009 season, when meteorological conditions were warmer. Contrarily, the overall severity of BBR in the same experimental field was significantly lower ($P < 0{\cdot}05$) in 2009 than in 2010 (8·2% compared with 21·7%, respectively; Calvo-Garrido *et al.* 2013), when weather conditions were cooler.

Meteorological conditions after veraison may have determined severity at harvest, whereas factors favouring opportunistic infections increase in this period as well (Barata *et al.* 2011), highlighting the importance of late season stages on sour rot epidemics.

Variations between years observed in hours $T > 30°C$ and days $T_{max} > 30°C$ were higher than in other meteorological variables measured (Table 1). Hours $T > 30°C$ were higher in 2009, while days $T_{max} > 30°C$ were more than the double in 2009 compared with 2010. These observations suggest these two variables as interesting parameters for future investigations on disease severity changes. Influence of high temperatures favouring sour rot compared with BBR is generally accepted. One of the possible causes could be the sensitivity of *Drosophila* spp. reproductive cycle to temperature, which can be six times faster at 25°C compared with 12°C (Galet 1999). Temperatures over 30°C may be also highly influent, as this is the temperature in which *B. cinerea* is not likely to grow and infect grape berries (Latorre *et al.* 2002), providing opportunities for the development of other fungal species, such as sour rot yeasts.

Nonetheless, more basic studies on the relationship of *B. cinerea*, sour rot yeasts and the effect of temperature are required. Some studies have evaluated fungal populations in berries with symptoms of sour rot (Gravot *et al.* 2001) or *B. cinerea* infection (Nisiotou and Nychas 2007; Barata *et al.* 2012a), but little is known about the effect of abiotic factors in the disease development on berries inoculated with *B. cinerea* and sour rot yeasts.

The effect of climatic conditions in disease shift among bunch rots has been studied in Australia for the relationship among *Botrytis*, ripe and bitter rot (Steel *et al.* 2010), which represents an interesting model of non-*Botrytis* rots. Laboratory and field research on bunch rot shift quantified the ability to infect the different pathogenic species and also related field severity to high temperatures over 30°C (Steel and Greer 2008; Steel *et al.* 2011).

This study reports biological control of sour rot for first time, after BBR control by *C. sake* and Fungicover applications had been previously observed (Calvo-Garrido *et al.* 2013), whereas it also improves knowledge of the meteorological conditions associated with sour rot in our region. Our findings increase the interest in *C. sake* plus FC applications as a double control strategy against the two main bunch rots in many viticultural regions. Nonetheless, further research is needed to fully understand sour rot epidemics and improve control of sour rot by these and other alternative treatments to synthetic fungicides, as fungicides are not providing reliable solutions, and a possible climate change scenario will favour the spread and intensity of non-*Botrytis* bunch rots, such as sour rot.

Materials and methods

Experimental field site (2009 & 2010)

Field experiments in both growing seasons were carried out in a commercial organic vineyard located in the Designation of Origin Costers del Segre (Lleida, Catalonia, Spain). Vines were trained into the traditional bush system of the region, and the grape cultivar used was Macabeu, which is characterized by large and very compact clusters (Fuster 2006).

Experimental design

Plots were distributed in a completely randomized block design, with four replicates per treatment. Each plot consisted of seven vines, the first and last vines were used as buffer vines, the next two vines were used for BCA population dynamics counts (Calvo-Garrido *et al.* 2013), and three vines were used to measure bunch rot development. All treatments against *Botrytis* bunch rot were applied at key vine phenological stages using a motorized backpack sprayer (WJR2225 model, Honda Motor Company Ltd, Frankfurt, Germany) with a 1 mm diameter nozzle and 15 bar pressure, spraying grape bunches until run-off.

Microbial antagonists and natural products

Candida sake CPA-1 strain was isolated from the surface of apples by University of Lleida-IRTA Research centre and was deposited at the 'Colección Española de Cultivos Tipo' (CECT-10817) in the University of Valencia, Burjassot, Spain. Cell production and formulation prior to field application were carried out according to the methods described by Cañamás *et al.* (2011).

Ulocladium oudemansii isolate HRU3 was applied using the formulated product BOTRY-Zen® (Botry-Zen Ltd, Dunedin, New Zealand). Chitosan was applied using the formulated product ARMOUR-Zen® (Botry-Zen Ltd).

Fungicover (BioDúrcal S.L., Granada, Spain) is a commercial formulation of derivatives of fatty acids in an aqueous alcoholic solution. It is used as a coating agent to improve *C. sake* survival on grape host tissues (Cañamás *et al.* 2011).

Bunch rot management programs and assessment

The bunch rot management programmes and the final concentrations of the active ingredients applied in 2009 and 2010 are detailed in Table 2 and Table 3, respectively.

At harvest, incidence and severity of sour rot were assessed on 50 bunches per replicate plot. Incidence was measured as the percentage of bunches with visual sour rot symptoms. Bunch rot severity was measured as the percentage berries per bunch presenting symptoms of sour rot.

Meteorological data

Temperature (T) and relative Humidity (RH) were logged at hourly intervals in both seasons using a weather station (Decagon Services Inc.) placed at the experimental field site. Daily average values were calculated and then used to obtain mean values for the postveraison period. Three variables were additionally calculated: T_{max} = mean value of the maximal daily temperatures; Hours $T > 30°C$ = number of hours in which temperature was over 30·0°C; Days $T_{max} > 30°C$ = number of days in which maximal temperature exceeded 30·0°C.

Statistical analysis

Analysis of variance was performed using JMP8 (SAS Institute Inc., Cary, NC, USA) for all data sets. Significant differences were determined using Student's t-LSD test ($P = 0·05$) for efficacy of treatments compared with untreated controls and Tukey test ($P = 0·05$) for comparisons of mean incidence and severity in untreated blocks between years.

Table 2 Field treatments with biologically based products in 2009 season

Treatment*	1–5% Flowering	80% Flowering	Prebunch closure	Veraison	21 days before harvest	7 days before harvest
Control	–	–	–	–	–	–
CS + FC	CS + FC	CS + FC	CS + FC	CS + FC	CS + FC	CS + FC
CS low + FC	CS low + FC	CS low + FC	CS low + FC	CS low + FC	CS low + FC	CS low + FC
AZ	AZ	AZ	AZ	AZ	AZ	AZ
BZ-AZ	BZ	BZ	BZ	AZ	AZ	AZ
BZ-(CS + FC)	BZ	BZ	BZ	CS + FC	CS + FC	CS + FC
(CS + FC)-AZ	CS + FC	CS + FC	CS + FC	AZ	AZ	AZ
FC	FC	FC	FC	FC	FC	

*Control: untreated; CS + FC: *C. sake* 5×10^7 CFU ml^{-1} + Fungicover 50 g l^{-1}; CS low + FC: *C. sake* 1×10^7 CFU ml^{-1} + Fungicover 50 g l^{-1}; AZ: chitosan 1·44 g l^{-1} + wetting agent 0·5 ml l^{-1} + 1·05 g l^{-1} of $NaHCO_3$; BZ: *Ulocladium oudemansii* $2·5 \times 10^6$ CFU ml^{-1} + wetting agent 0·5 ml l^{-1}; FC: Fungicover 50 g l^{-1}.

Table 3 Field treatments with *Candida sake* CPA-1 and Fungicover in 2010 season

Treatment*	80% Flowering	Prebunch closure	Veraison	21 days before harvest
Control	–	–	–	–
CS + FC × 4	CS + FC	CS + FC	CS + FC	CS + FC
FC × 4	FC	FC	FC	FC
CS + FC × 2	CS + FC	CS + FC	–	–
FC × 2	FC	FC	–	–
CS + FC25 × 4	CS + FC25	CS + FC25	CS + FC25	CS + FC25
FC25 × 4	FC25	FC25	FC25	FC25
CS + FC25 × 2	CS + FC25	CS + FC25	–	–
FC25 ×2	FC25	FC25	–	–

*Control: untreated; CS: *C. sake* 5×10^7 CFU ml^{-1}; FC: Fungicover 50 g l^{-1}; FC25: Fungicover 25 g l^{-1}; ×4: four applications from flowering to harvest; ×2: two applications in early season (80% flowering and prebunch closure).

Acknowledgements

Authors are grateful to the University of Lleida for Carlos Calvo Garrido PhD grant and organic production research project, the European Union (INTERREG RED-BIO project) for their financial support and grant. Also to the Catalan Government and Plant and Food Research for Dr. Elmer's travel assistance.

References

Barata, A., Pais, A., Malfeito-Ferreira, M. and Loureiro, V. (2011) Influence of sour rotten grapes on the chemical composition and quality of grape must and wine. *Eur Food Res Technol* **233**, 183–194.

Barata, A., Malfeito-Ferreira, M. and Loureiro, V. (2012a) The microbial ecology of wine grape berries. *Int J Food Microbiol* **153**, 243–259.

Barata, A., Santos, S.C., Malfeito-Ferreira, M. and Loureiro, V. (2012b) New insights into the ecological interaction between grape berry microorganisms and drosophila flies during the development of sour rot. *Microb Ecol* **64**, 416–430.

Calvo-Garrido, C., Elmer, P.A.G., Viñas, I., Usall, J., Bartra, E. and Teixidó, N. (2013) Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1. *Plant Pathol* **62**, 510–519.

Cañamás, T.P., Viñas, I., Torres, R., Usall, J., Solsona, C. and Teixidó, N. (2011) Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. *Biol Control* **56**, 150–158.

Dimakopoulou, M., Tjamos, S.E., Antoniou, P.P., Pietri, A., Battilani, P., Avramidis, N., Markakis, E.A. and Tjamos, E.C. (2008) Phyllosphere grapevine yeast Aureobasidium pullulans reduces Aspergillus carbonarius (sour rot) incidence in wine-producing vineyards in Greece. *Biol Control* **46**, 158–165.

Fermaud, M., Gravot, E. and Blancard, D. (2002) Sour rot in the vineyards of Bordeaux. II - transmission of pathogenic microorganisms by fruit flies. *Phytoma* **547**, 41–44.

Fuster, P.M.C. (2006) *Variedades de vid: registro de Variedades Comerciales*, 2nd edn. Madrid: Ministerio de Agricultura, Pesca y Alimentacion.

Galet, P. (1999) *Précis de Pathologie Viticole*. Montpellier: Lavoisier.

Gravot, E., Blancard, D., Fermaud, M., Lonvaud, A. and Joyeux, A. (2001) Sour rot. I: etiology. Research into the causes of this form of rot of grapes in Bordeaux vineyards. *Phytoma* **543**, 36–39.

Guerzoni, E. and Marchetti, R. (1987) Analysis of yeast flora associated with grape sour rot and of the chemical disease markers. *Appl Environ Microbiol* **53**, 571–576.

Huber, C., McFadden-Smith, W. and Inglis, D. (2011) Management and etiology of grape sour rot in the Niagara region. *Phytopathology* **101**, S259–S259.

Latorre, B.A., Rioja, M.E. and Lillo, C. (2002) Effect of temperature on flower and berry infections caused by *Botrytis cinerea* on table grapes. *Cien Inv Agr* **29**, 145–151.

Ligorio, A., Platania, G., Schena, L., Castiglione, V., Pentimone, I., Nigro, F., Silvestro, I.D. and Ippolito, A. (2007) Control of table grape storage rots by combined applications of antagonistic yeasts, salts and natural substances. In *Novel Approaches for the Control of Postharvest Diseases and Disorders*, ed. Congress, I. pp. 124–128. Bologna, Italy: CRIOF, University of Bologna.

Marchetti, R., Guerzoni, M.E. and Gentile, M. (1984) Research on the etiology of a new disease of grapes: sour rot. *Vitis* **23**, 55–65.

Nigro, F., Schena, L., Ligorio, A., Pentimone, I., Ippolito, A. and Salerno, M.G. (2006) Control of table grape storage rots by pre-harvest applications of salts. *Postharvest Biol Tec* **42**, 142–149.

Nisiotou, A.A. and Nychas, G.J.E. (2007) Yeast populations residing on healthy or Botrytis-infected grapes from a vineyard in Attica, Greece. *Appl Environ Microbiol* **73**, 2765–2768.

Oriolani, E.J., Rodriguez-Romera, M. and Combina, M. (2009) Parasitic complex of acid rot bunches of grapes, in Mendoza and San Juan, Argentina. *In Abstract Book of the XV Latin-American Congress and XVIII Chilean Congress of Plant Pathology*. Santiago, Chile, Asociación Latinoamericana de Fitopatología and the Sociedad Chilena de Fitopatología. p.204.

Rooney-Latham, S., Janousek, C.N. and Gubler, W. (2007) The use of various pre-harvest practices for the management of "sour rot" and "Non-Botrytis Slip Skin" of Red Globe table grapes. *Phytopathology* **97**, S101–S101.

Sivilotti, P., Malossini, G., Stocco, M., Colussi, G., Bregant, F., Gallas, A., Miletti, R. and Fabbro, A. (2011) Techniques of vine defoliation and bunch rot. *Inf Agr* **67**, 67–71.

Spring, J.L. and Viret, O. (2009) Influence of thinning methods on yield, bunch morphology, grey and sour rot, and wine quality of Pinot noir. *Revue suisse Vitic Arboric Hortic* **41**, 95–101.

Stapleton, J.J. and Grant, R.S. (1992) Leaf removal for nonchemical control of the summer bunch rot complex of wine grapes in the San-Joaquin valley. *Plant Dis* **76**, 205–208.

Steel, C.C. and Greer, D.H. (2008) Effect of climate on vine and bunch characteristics: Bunch rot disease susceptibility. In *Proceedings of the International Symposium on Grape Production and Processing*. Acta Hort eds. Adsule, P.G., Sawant, I.S. and Shikhamany, S.D. pp.253–262.

Steel, C.C., Greer, D.H., Samuelian, S.K. and Savocchia, S. (2010) Effects of climate and mixed bunch rot infection on *Botrytis cinerea* (grey mould) of grapes: implications for global climate change and disease management. In *Abstract Book of the XV International Botrytis symposium*. Cadiz, Spain, University of Cadiz. p.57.

Steel, C.C., Greer, L.A., Savocchia, S. and Samuelian, S.K. (2011) Effect of temperature on *Botrytis cinerea*, *Colletotrichum acutatum* and *Greeneria uvicola* mixed fungal infection of *Vitis vinifera* grape berries. *Vitis* **50**, 69–71.

Wei, Y., Li, X., Cai, J., Zhang, X., Liu, Z., Shang, Q. and Zhao, X. (2011) Identification of yeasts associated with grape sour rot in the north of China. *Phytopathology* **101**, S189–S189.

CAPÍTULO 4

Mode of action of a fatty acid-based natural product to control of *Botrytis cinerea* in grapes

C. Calvo-Garrido, P.A.G. Elmer, F.J. Parry, I. Viñas, J. Usall, R. Torres, R.H. Agnew and N. Teixidó

Enviado a: ***Journal of Applied Microbiology***

Mode of action of a fatty acid-based natural product to control *Botrytis cinerea* in grapes

Authors

C. Calvo-Garrido [1], P.A.G. Elmer [2], F.J. Parry [2], I. Viñas [1], J. Usall [3], R. Torres [3], R.H. Agnew[4] and N. Teixidó [3]*

[1] Food Technology Department, Lleida University, XaRTA-Postharvest, Agrotecnio Center, Rovira Roure 191, 25198 - Lleida, Catalonia, Spain

[2] The New Zealand Institute for Plant & Food Research Limited, Ruakura Research Centre, Private Bag 3230, Waikato Mail Centre, Hamilton, 3240, New Zealand

[3] IRTA, XaRTA-Postharvest, 191 Rovira Roure, 25198-Lleida, Catalonia, Spain

[4] The New Zealand Institute for Plant & Food Research Limited, Marlborough Wine Research Centre, PO Box 845, Blenheim, New Zealand

Abstract

Aims: To investigate the efficacy and mode of action of the fatty acid-based product Foodcoat® (FC) against *B. cinerea*.

Methods and Results: *In vitro*, *in vivo* and field experiments were carried out to investigate the effect of different concentrations of FC on *B. cinerea* germination and infection of grape leaves and berries, using three selected isolates and comparing results with those achieved by the commercialized product Protector[HML] (PRT). Furthermore, the effect of field applications of FC on the grape berry microbiota was investigated. FC reduced *B. cinerea* germination and grape berry severity by up to 54% and 96% respectively, compared with the untreated controls.

Conclusions: Foodcoat demonstrated efficacy that was equal or greater than the registered product, PRT. A multiple mode of action was hypothesized for FC suppression of *B. cinerea*, including: inhibition of germination and germ tube alteration, protection of host green tissues and enhancement of the natural yeast populations on the berry surface.

Significance and Impact of Study: The efficacy of two fatty acid formulations has been quantified and their modes of action described, suggesting these products as valuable additives for biological control agents. This is also the first report of a fatty acid-based product stimulating natural yeast populations on grape berries.

Keywords

Lauric acid, estearic acid, palmitic acid, potassium soap, *Vitis vinifera*, adjuvant, biological control, epiphytic population

Introduction

The necrotrophic fungus, *Botrytis cinerea* is an important plant pathogen affecting production quantity and quality in a wide range of crops in mostly every climatic region (Elad *et al.* 2004). In grapes, *B. cinerea* is the causal agent of *Botrytis* bunch rot (BBR), and is responsible for significant economic losses in vineyards world-wide, especially when climatic conditions are favorable for disease epidemic development (Elmer and Michailides 2004). Conventional approaches to BBR control includes synthetic fungicides applications, while canopy management, such as leaf removal to improve air flow and reduce the risk of *B. cinerea* infection periods is becoming more important (Wurms *et al.* 2011; Tardaguila *et al.* 2012). However, this cultural practice, especially when used in the absence of other strategies, is not sufficient to effectively and consistently reduce losses caused by BBR. In addition, dependence upon fungicides has fallen out of favor over the last decade due to several factors including; frequent reports of resistance development by *B. cinerea* to newly introduced fungicides (Zhao *et al.* 2010; Walker *et al.* in press), damage to the environment (Komárek *et al.* 2010), and the persistence of some fungicide residues throughout the winemaking process (Cus *et al.* 2010). As a consequence, European and non-European winegrowing countries have adopted more restrictive policies regarding fungicide use. The replacement of old technology active ingredients, such as captan and reduction of newly introduced synthetic fungicides has become an important priority for wine grape producers globally, which in turn has stimulated research into alternative treatments for BBR control in vineyards, including biological control agents (BCAs) and natural products (NPs) (Elmer and Reglinski 2006).

A natural product is broadly defined as an active ingredient that is derived from a plant, animal or microbial source that brings about a reduction in disease development mediated through stimulating plant defenses, direct anti-fungal activity and/or biofilm formation (Tripathi and Dubey 2004; Romanazzi *et al.* 2012).

Fatty acids are NPs that have received special attention due to their antimicrobial properties, including the ability to control human and foodborne pathogens (Ricke *et al.* 2005; Karlova *et al.* 2010; Desbois 2012). In contrast, the screening and application of these compounds for plant protection purposes has been less studied (Liu *et al.* 2008). Nonetheless, fatty acid compounds have shown efficacy against insect pests (Ma and Gu 2003; Penaflor *et al.* 2006) and, in addition, several studies have reported effective suppression of important phytopathogenic fungi including *B. cinerea* by this kind of products (Rihakova *et al.* 2001; Wang *et al.* 2002; Walters *et al.* 2004; Karlova *et al.* 2010). Other fatty acid derivatives, such as oxygenated fatty acids or bioconverted fatty acids, have also shown antimicrobial activity, and represent a potentially new group of NPs for the future development of alternative products for the control of *B. cinerea* and other phytopathogenic fungi (Hou and Forman 2000; Hou 2008; Bajpai *et al.* 2009).

In addition to their anti-fungal properties, these NPs present other practical advantages, which include that they may be applied close to and at harvest at a time when synthetic agrichemicals may leave a residue on the fruit and in the wine, whereas their abundance in nature and relative ease of extraction (Ruiz-Rodriguez *et al.* 2010) ensures reduced production costs compared to other NPs, making them attractive as additives for BCA applications.

Moreover, fatty acid-based products have been used as useful adjuvants for synthetic pesticides, other natural products or BCAs (Alzaemey *et al.* 1993; Coleman and Penner 2008; Cañamás *et al.* 2011). The commercial product Protector^{HML} (PRT; Henry Manufacturing Limited, New Zealand) is based on potassium salts of fatty acids and manufactured in New Zealand with label claims for BBR and powdery mildew control (http://www.henrymanufacturing.co.nz/products/protectorhml/). It has also been field evaluated in New Zealand as an adjuvant for selected BCAs. For example, when combined with *Ulocladium oudemansii*, coverage of immature developing grape bunches and colonization of necrotic bunch trash by this BCA was significantly improved (Kirsten Bevin, BotryZen Ltd, *pers. com.*). This type of approach, combining BCAs and low dose NPs for improved coverage and efficacy represents a significant advancement in the development of alternative, cost effective, biologically based strategies for disease control.

The commercial product Foodcoat® (FC; Domca S.L., Spain) is a preparation based on derivatives of fatty acids in an aqueous-alcoholic solution and is classed as an edible coating agent for fruit and vegetables, with a label claim for improving fruit firmness and visual appearance during post harvest and after shelf life. A less concentrated formulation of the same product, Fungicover® Base (Biodúrcal S.L., Spain), was used as an adjuvant for field applications of the BCA *Candida sake* CPA-1, to increase the field survival of *C. sake* populations on grapevine tissues in Catalan vineyards (Cañamás *et al.* 2011). Further studies also demonstrated that FC alone reduced *B. cinerea* secondary inoculum (Calvo-Garrido *et al.* in press) and BBR incidence and severity at harvest (Calvo-Garrido *et al.* 2013). Nonetheless, control of *B. cinerea* infection by FC has not been quantified under controlled conditions and the mode of action against *B. cinerea* remains unclear.

The objectives of this work were a) to formulate a hypothesis on the mode of action of the fatty acid-based product FC, based upon the results of controlled environment studies and field findings, and b) to quantify the reduction in *B. cinerea* severity on grapevine tissues with different FC treatments, compared with the commercialized product PRT.

Materials and methods

Fatty acid-based natural products

The reference fatty acid-based NP used in this study was Foodcoat® FR drencher DMC (Domca S.L., Spain). Its active ingredients are: lauric acid, palmitic acid and estearic acid, esterified with glycerine and sugars. The precise concentrations of each component are a trade secret. The final formulation is a mixture of glycerides, sucrose esters and sucroglycerides in an aqueous emulsion containing 4000 ppm of ethanol. Manufacturers state that FC slows down maturation and reduces weight loss during post-harvest storage of fruit by reducing respiratory activity and ethylene production, while it is also reported to have fungistatic effects (http://www.domca.com/images/stories/PDF_ing/poscosecha/foodcoat%20drencher.pdf). The recommended dose when applied as an edible food coating ranges from 2% (v v^{-1}) to 4 % (v v^{-1}).

The fatty acid-based product, Protector^{HML} (PRT; Henry manufacturing Limited, New Zealand) was used in the bioassays in order to compare FC with another fatty acid-based NP used in vineyards and orchards in New Zealand. Its composition is based on complexes of potassium salts of fatty acids and recommended doses are 2% (v v^{-1}) for BBR and powdery mildew control and 0·5% (v v^{-1}) for its use as adjuvant (http://www.henrymanufacturing.co.nz/products/protectorhml/).

B. cinerea inoculum

In order to evaluate the effect of FC on a range of *B. cinerea* phenotypes, three *B. cinerea* isolates from the Plant & Food Research Institute (PFR), Ruakura Research Centre culture collection, were used in these studies. Based upon preliminary assays on grapevine tissues, these isolates were classed as moderate to highly pathogenic on leaves (Iso2 and Iso6) and berries (Iso2 and Iso7; data not shown). Iso2 was sensitive to benzimidazole and dicarboximide fungicides, Iso6 was characterized as resistant to benzimidazole and sensitive to dicarboximide, whereas Iso7 was resistant to benzimidazole and dicarboximide fungicides.

Stored cultures were maintained at -70 °C in 15% glycerol (v v^{-1}) and then, depending on the assay, subcultured onto 100% or 2·5% PDA (Scharlab S.L., Spain) 90 mm Petri dishes in the dark. After 7-10 days incubation, fresh cultures of the isolates were used a) to obtain 5 mm diameter mycelial plugs from the edge of the culture, or b) to harvest conidia by flooding each culture with sterile distilled water (SDW) containing Tween 80 (0·01% v v^{-1}) and then filtering the subsequent suspensions through sterile cell strainers (Falcon, 100-µm mesh), and adjusting the concentration, as required, with the aid of a haematocytometer.

Grapevine tissues for in vivo assays

Two sources of grapevine (*Vitis vinifera* L.) tissues cv. Chardonnay were used and were collected from experimental vines, located at the PFR, Ruakura campus (Hamilton, New Zealand). Fully expanded leaves were collected from five year old potted vines grafted onto on SO4 rootstock, which had been grown in an unheated, ventilated glasshouse. Grape bunches cv. Chardonnay were collected from a small, six year old experimental vineyard grown on SO4 rootstock that received fortnightly applications of sulfur for powdery mildew control. Grape bunches cv. Sauvignon blanc were taken from a commercial vineyard in the Marlborough winegrowing region of NZ, then carefully packed and couried to PFR, Ruakura research centre. The five year old vines were of a mass selected clone grafted onto 101-14 rootstock and rows were spaced 2·7 m apart with vines 1·8 m apart within the row. The whole vineyard received one application of fluazinam on flowering (12/12/2010) and one application of cyprodinil + fludioxonil at pre bunch closure (12/01/2011), both applied at commercial application rates provided by manufacturer. For the investigation of FC efficacy against *B. cinerea* on grape berries, both Chardonnay and Sauvignon blanc bunches of similar maturity and free of any defects were visually selected after veraison.

Effect of Foodcoat on B. cinerea germination

Germination of *B. cinerea* conidia was measured in solutions of SDW with different concentrations of the fatty acid-based products (0·5, 1, 2·5 and 5 % v v^{-1} of FC; and 0·5 and 2 % v v^{-1} of PRT). The six treatments were compared with an untreated control (SDW). Three test tubes (replicates) of each treatment were prepared for each isolate (Iso2, Iso6 and Iso7). The final conidia concentration in the tubes was adjusted to $5x10^4$ conidia ml^{-1}. After 30 minutes contact time with *B. cinerea* conidia, three 10 μL aliquots per isolate * treatment * replicate were plated onto PDA plates, then incubated at 20 °C in the dark for 10 hours and germination terminated by adding 1 ml of ammonia solution (30% NH_3; BDH Analar, VWR International Pty. Ltd., Australia) to a filter paper disc (5 mm diameter) fixed on the Petri dish lid and then closing the plate with the lid. Then, three PDA plugs (5mm diameter) corresponding to the three aliquots were mounted onto a microscope slide and observed under the microscope (MZ 16F, Leica Microsystems GmbH, Germany). On each PDA plug, 50 conidia were counted and germination was considered to have occurred if the germ tube was equal to, or greater than the diameter of the spore. The number of germinated conidia from 150 conidia per isolate * treatment * replicate were expressed as percent germinated conidia (%). Microscopic photography was taken of germinating and non-germinated conidia using a Leica DFC420 (Leica Microsystems GmbH, Germany) digital camera attached to the microscope (40x).

Effect of Foodcoat on B. cinerea mycelial infection of Chardonnay leaves

Fully expanded Chardonnay leaves (clone UDC15) were removed from the potted grapevines described above and the following treatments applied: SDW only (Control), FC at 0·5, 1, 2·5 and 5 % (v v^{-1}) and 0·5 and 2 % (v v^{-1}) of PRT using a hand held 1-liter pump sprayer. After one hour of air drying, two 5 mm diameter mycelial plugs of each isolate (Iso2 and Iso6) that had been grown on 2·5% PDA medium in the dark (to encourage mycelial growth but delay conidial formation), were placed with the *B. cinerea* mycelium plug facing down on the adaxial surface of each grape leaf. They were positioned symmetrically, with two plugs on either side of the midrib vein and taking care to avoid placement over secondary veins. Inoculated leaves were then placed in high humidity chambers (HHCs) with the cut end of the leaf pedicel embedded in a carefully folded sterile paper towel that had been thoroughly wetted with SDW. The high humidity chambers consisted of a shallow tray (50 cm x 30 cm) with a surface sterilized plastic grid (40 cm x 25 cm) placed over two sterile paper towels that had been moistened with 50 ml of SDW, then introduced in a polyethylene plastic bag.

All treated leaf samples were incubated at room temperature (22 ± 1 °C), for eight days. Typical *B. cinerea* lesions were measured with the aid of electronic calipers (Absolute Digimatic Series 500, Mitutoyo Asia Pacific Pte. Ltd., Japan), by measuring lesion diameter (mm) along each axis, perpendicular to each other. Mean of the two measured diameters (mm) was calculated for the lesion produced by each inoculum plug. The mean lesion diameter produced by the two plugs of the same isolate on the same leave were considered as a replicate unit of each treatment * isolate. In total, there were six replicates per treatment.

Effect of Foodcoat on B. cinerea infection of grape berries

The ability of FC and PRT to reduce *B. cinerea* infection of grape berries was evaluated in two different experiments: 1) infection of intact berries inoculated with 5 mm diameter mycelial plugs that had been grown on 100% PDA, and 2) infection of wounded berries inoculated with a *B. cinerea* conidial suspension (5 x 10^4 conidia ml^{-1}).

In both experiments, there were five berries per replicate and there were six replicates for each treatment * cultivar * isolate. The *B. cinerea* isolates used in the grape berry inoculation studies were Iso2 and Iso7.

Wounding was carried out by gently rubbing the grape berry surface with a small strip of sterile sandpaper (P220 grit number; Norton Abrasives Pty. Ltd., Australia). Wounds observed under a stereo binocular microscope showed that this wounding treatment created wounds that were approximately 5 mm in diameter on the berry cuticle and were not so deep as to expose the berry flesh.

Chardonnay and Sauvignon blanc berries (sugar content: Mean ± SE = 21·4 ± 0.06 and 19·3 ± 0.26 °Brix, respectively) from research plots that were unsprayed from bunch closure were cut from the bunch with the pedicel attached. All berries (720 of each cultivar) were then washed twice in a sterile beaker with tap water placed in a rotary shaker (Innova 2300, New Brunswick Scientific Co. Inc., USA) at 110 rpm for ten and five min, respectively. Fresh tap water was used for each washing process. After air drying in a laminar flow hood, half of the berries were wounded as described above. Wounded and unwounded berries were then treated with: water (Control) and fatty acid-based solutions adjusted to 0·5, 1, 2·5 and 5 % v v^{-1} of FC and 2 % v v^{-1} of PRT, by dipping each treatment replicate (five berries) in a 1L plastic beaker for 30 seconds. Treated berries were then placed onto sterile plastic grids in a laminar flow hood and allowed to dry. There were 6 replicates per treatment in total.

Unwounded berries were inoculated with 5 mm diameter mycelial plugs of each isolate (Iso2 and Iso7), with a single plug per berry. Wounded berries were inoculated with 10 µL of a conidial suspension (5 x 10^4 conidia ml^{-1}) of each *B. cinerea* strain (Iso2 and Iso7). A 5 mm diameter sterile plastic ring was placed around the wound site, prior to inoculation, and sealed in place with paraffin wax, to avoid run-off and loss of the *B. cinerea* suspensions.

Treated and inoculated berries were placed into HHCs (as described earlier) and incubated at room temperature for seven days prior to results assessment. Severity of *B. cinerea* infection on each berry was measured by visually scoring the percentage of the grape berry surface with typical *B. cinerea* berry rot symptoms.

Effect of Foodcoat on grape berry fungal microbiota

The effect of FC applications on the grape berry surface fungal populations was examined, in order to establish whether the effect of FC on *B. cinerea* was mediated via the grape berry microbiota. Filamentous fungi, yeasts and yeast-like fungi from the grape berry surface were recovered from bunches that had received three applications of FC applied at 0 (control), 0·5, 1

or 2·5% (v v^{-1}) between veraison and harvest. Field applications were applied in a separate plot of the previously described commercial vineyard used to obtain berries for in vivo assays (cv. Sauvignon blanc), located in the Marlborough winegrowing region of New Zealand. Treatments were applied at a 500 l ha^{-1} rate with a motorized backpack sprayer (model 425, Solo Inc., New Zealand). The three applications of FC were carried out at veraison (22/02/2011), veraison + 3 weeks (17/03/2011), veraison + 5 weeks (30/03/2011). There were six replicates in a Randomized Blocks Design, where replicate plots consisted of four vines (7·2 m length).

At harvest, four bunches per replicate plot were randomly selected. In the laboratory, five apparently sound berries were sampled from each bunch (two from the top of the bunch, 2 from the middle and 1 from the bottom) using sterile clippers and leaving 2-3 mm of the pedicel attached. The 20 berries per replicate plot were weighted and placed into a 250 ml Erlenmeyer flask with 50 ml of phosphate buffer, then shaken for 10 min in a rotary shaker (Innova 2300, New Brunswick Scientific Co. Inc., USA) at 150 rpm and then sonicated for 10 min in an ultrasonic bath (Sonorex Digital 10P, Bandelin Electronic GmbH & Co., Germany). After serial dilutions of the washing solution, 100-µL aliquots were spread plated onto Semi-Selective Yeast Agar (SSYA+: bacto yeast nitrogen base, 6·7 g l^{-1}; dextrose, 10·0 g l^{-1}; bacto agar, 18·0 g l^{-1}; iprodione, 0·01 g l^{-1}; dichloran, 0·00267 g l^{-1} and chloramphenicol, 0·10 g l^{-1}) and Rose Bengal Agar (RBA; Oxoid Ltd., UK), to quantify yeast and yeast-like fungi, or filamentous fungi, respectively. Duplicate plates of each serial dilution were incubated at 25 °C in the dark for 48 hours (SSYA+) or six days (RBA) in order to determine population counts. Colonies were visually classified into white yeasts, pink yeasts, yeast-like fungi or filamentous fungi based on their morphological characteristics. Population count data was expressed as colony forming units (CFU) g^{-1} of grape berries.

Statistical analysis

Data were analysed by ANOVA using JMP-8 software (SAS Institute Inc.). Significant differences of treatments compared to the untreated controls were determined according to Student's t LSD test (P = 0·05) for all experiments. Berry surface fungal population count data (CFU g^{-1}) were log-transformed, prior to ANOVA, in order to stabilize the variance.

Results

Effect of Foodcoat on B. cinerea germination

Conidia of three *B. cinerea* isolates were immersed in solutions with a range of concentrations of FC and PRT for 30 minutes and then 10µl aliquots placed onto PDA plates. Percentage of germinated *B. cinerea* untreated conidia after 10 hours of incubation was 97·2% (Iso2), 97·0% (Iso6) and 95·7% (Iso7), as shown in Fig. 1. The low concentrations of FC or PRT (0·5%) did not significantly reduce (P < 0·05) conidial germination and only the higher concentrations of the products (FC at 2·5 and 5%; PRT at 2%) significantly reduced (P < 0·05) the percentage of conidia that germinated across all three isolates. Reductions of the germination percentage

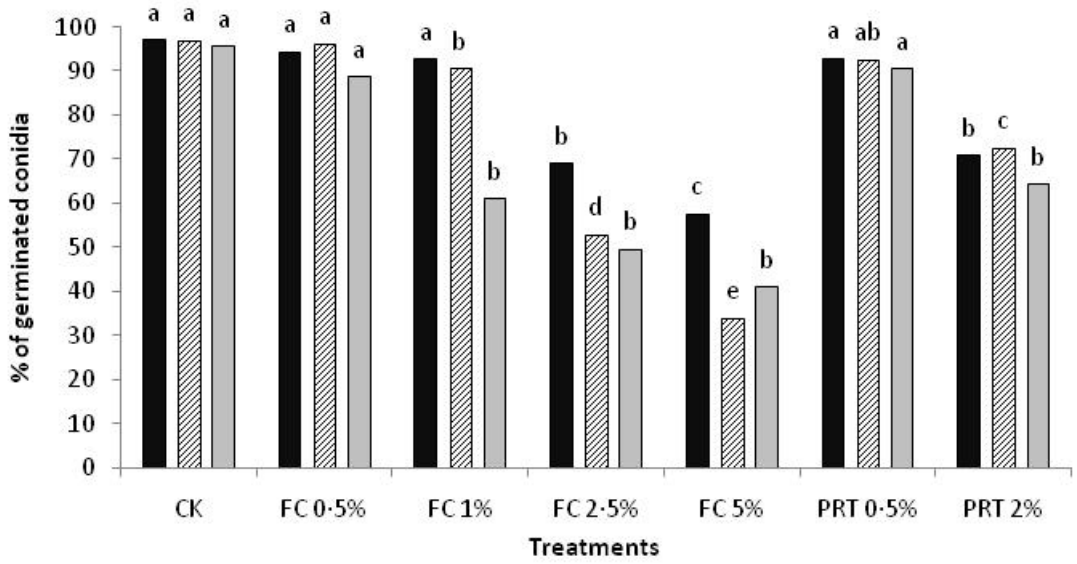


Figure 1 Effect of fatty acid based product Foodcoat (FC) on conidial germination of three isolates of *Botrytis cinerea* after 10 hours incubation at 20 ^{0}C on PDA medium. Iso2 ( ), Iso6 ( ) and Iso7 ( ) conidia were presoaked in water (CK) or different concentrations of Foodcoat® (FC; 0·5, 1, 2·5, and 5 % v v^{-1}) and Protector^{HML} (PRT; 0·5, 2 % v v^{-1}) for 30 minutes and then plated onto PDA. Values are means of three replicates per treatment. Means linked with the same letter for each isolate are not significantly different (P < 0·05), according to LSD test.

(mean of three isolates) were 40% (FC 2·5%), 54% (FC 5%) and 28% (PRT 2%), compared to the untreated control (CK). However, there was differential sensitivity between the isolates, as showed the significant interaction isolate*treatment (P < 0·05; data not shown). Therefore, data of the three selected isolates could not be pooled for the statistical analysis.

Significant differences in isolate sensitivity were evidenced, since the FC 1% treatment significantly reduced (P < 0·05) germination of Iso6 and Iso7, while germination of Iso2 was unaffected, compared to the untreated controls. In addition, in the FC 1% treatment, germination reduction was significantly higher in Iso7, compared to Iso2 and Iso6. When conidia were exposed to higher doses of FC (2·5% and 5%), germination percentage was lower in Iso6 and Iso7 than in Iso2, although differences were not significant.

Conidial treatment with FC not only reduced *B. cinerea* conidial germination but also affected germ tube morphology (Fig. 2). Germinated conidia in the water control (Fig. 2a) typically had germ tubes that were long and straight, while the conidia maintained a typical oval-lemon shape. However, after treatment with FC 1% and FC 2·5%, conidia appeared to be surrounded by a dense matrix, containing larger sized particles, which are likely to be part of the FC formulation (Fig. 2b, c). The density of the matrix and the conglomerate number increased as FC concentration increased. Non-germinated conidia treated with FC 2·5% were larger and rounded, while the germ tubes of germinated conidia (Fig. 2b, c) were typically shorter and more swollen, compared to the untreated control, indicating morphological changes in treated conidia. The described effect was also observed in FC 5% samples (data not shown), whereas PRT treated conidia and germ tubes did not show morphological alterations.

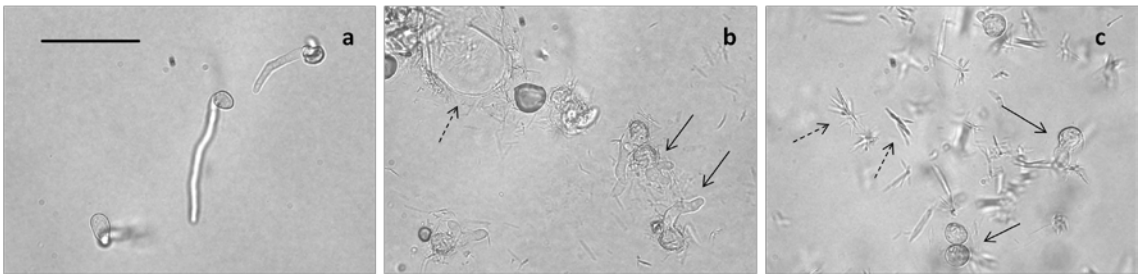


Figure 2 Germination of *Botrytis cinerea* (Iso7) conidia treated with Foodcoat (FC) after 10 hours of incubation at 20 ºC on PDA medium. Conidia were suspended in concentrations of Foodcoat® at a) 0% v v^{-1}, b) 1% v v^{-1} and c) 2·5% v v^{-1} for 30 minutes prior to incubation on PDA. Solid arrows indicate conidia and germ tubes with characteristic swollen and altered morphology. Dotted line arrows indicate Foodcoat conglomerates. (Bar = 0·03 mm).

Effect of Foodcoat on B. cinerea mycelial infection of Chardonnay leaves

The severity of *B. cinerea* infection of Chardonnay leaves after eight days of incubation in high HHCs is presented in Fig. 3. There was no significant interaction ($P < 0·05$) detected between isolate lesion size and treatments. Therefore, the lesion severity data for leaves inoculated with Iso2 and Iso6 were pooled. All FC treatments significantly reduced ($P < 0·05$) lesion diameter in inoculated Chardonnay leaves by 52% (FC 1%) to 75% (FC 5%) compared with water treated control (CK). PRT reduced lesion diameter by 66% (PRT 0·5%) and 76% (PRT 2%), indicating that both fatty acid products were capable of reducing *B. cinerea* lesion development. Dose response was not significant, although FC 5% and PRT 2% treatments, corresponding to the most elevated dose of each product, achieved the highest lesion diameter reduction.

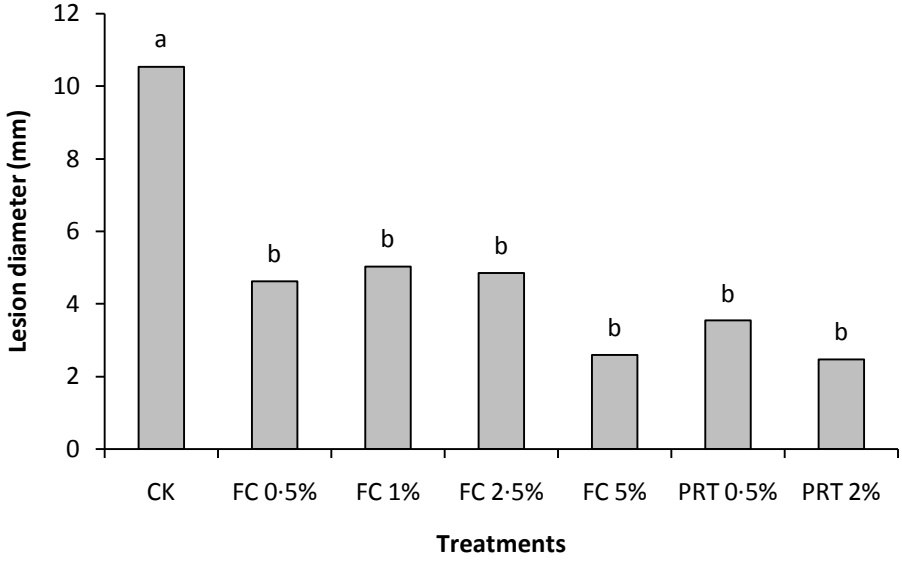


Figure 3 Effect of fatty acid based product Foodcoat (FC) on lesion diameter caused by inoculation of Chardonnay leaves with *B. cinerea* mycelial plugs, after eight days of incubation at 22 ± 1 ºC. Young leaves were sprayed with water (CK) or different concentrations of Foodcoat® (FC; 0·5, 1, 2·5, and 5 % v v^{-1}) and Protector^{HML} (PRT; 0·5, 2 % v v^{-1}). Leaf inoculation was carried out with 5mm mycelium plugs from the margin of seven-day old cultures on 2·5% PDA. Values are means of six replicates of each isolate tested. Mean values linked by the same letter are not significantly different ($P < 0·05$) according to LSD test.

Effect of Foodcoat on B. cinerea infection of sound grape berries

The effect of FC concentration on the severity of *B. cinerea* infection of unwounded Chardonnay and Sauvignon blanc berries was measured by artificially inoculation with *B. cinerea* mycelial plugs (Fig. 4). There were significant interactions ($P < 0{\cdot}05$) detected between cultivar and isolate, cultivar and treatment, and isolate by treatment, therefore, results for each isolate and cultivar are presented separately. In general, FC and PRT treatments significantly reduced *B. cinerea* rot severity ($P < 0{\cdot}05$) in both cultivars against both isolates with the exception of the low FC concentration (FC 0·5%) on Chardonnay berries inoculated with Iso2 (Fig. 4a).

As FC concentration increased, *B. cinerea* severity significantly declined, suggesting a dose response. In Chardonnay (Fig. 4a, b), there were significant differences ($P < 0{\cdot}05$) between the FC 0·5% treatment and the FC 2·5% and FC 5% treatments when challenge inoculated with Iso7. When inoculated with Iso2, significant differences were evidenced only between FC 0·5% and FC 5%. The FC 2·5% and FC 5% treatments reduced severity by 46% and 51% (Iso2; Fig. 4a) and by 90% and 96% (Iso7; Fig. 4b), compared to untreated control (CK).

The dose effect of FC concentration was also observed on Sauvignon blanc berries. The FC 5% treatment presented significantly lower ($P < 0{\cdot}05$) severity than the rest of FC treatments when berries were inoculated with Iso2, whereas both FC 2·5% and FC 5% treatments significantly reduced severity more than the lower concentrations (FC 0·5% and FC 1%) when challenge inoculated with Iso7 (Fig. 4c, d). The reduction of *B. cinerea* rot severity by FC treatments at 2·5% and 5% were, 44% and 92% (Iso2) and 84% and 97% (Iso7) respectively.

For both evaluated isolates, *B. cinerea* rot severity on untreated Sauvignon blanc berries was significantly higher ($P < 0{\cdot}05$), compared with Chardonnay, indicating that this variety was more susceptible to the two isolates of *B. cinerea* used.

Overall, *B. cinerea* rot severity after inoculation with Iso7 was significantly lower ($P < 0{\cdot}05$) compared with Iso2 on Chardonnay berries suggesting that Iso7 was less aggressive. However, the difference between isolates was not detected on Sauvignon blanc.

Further analysis of the significant cultivar*treatment and cultivar*isolate interactions ($P < 0{\cdot}05$) showed that differential sensitivity of the evaluated cultivars and differences between Iso2 and Iso7 were not observable on berries treated with the higher FC concentrations (FC 2·5% or FC 5%).

PRT at a 2% rate significantly reduced severity of *B. cinerea* mycelial infection for both cultivars and isolates tested. Reduction achieved was similar to those of FC 2·5% treatment, except for Sauvignon berries inoculated with Iso7 (Fig. 4d).

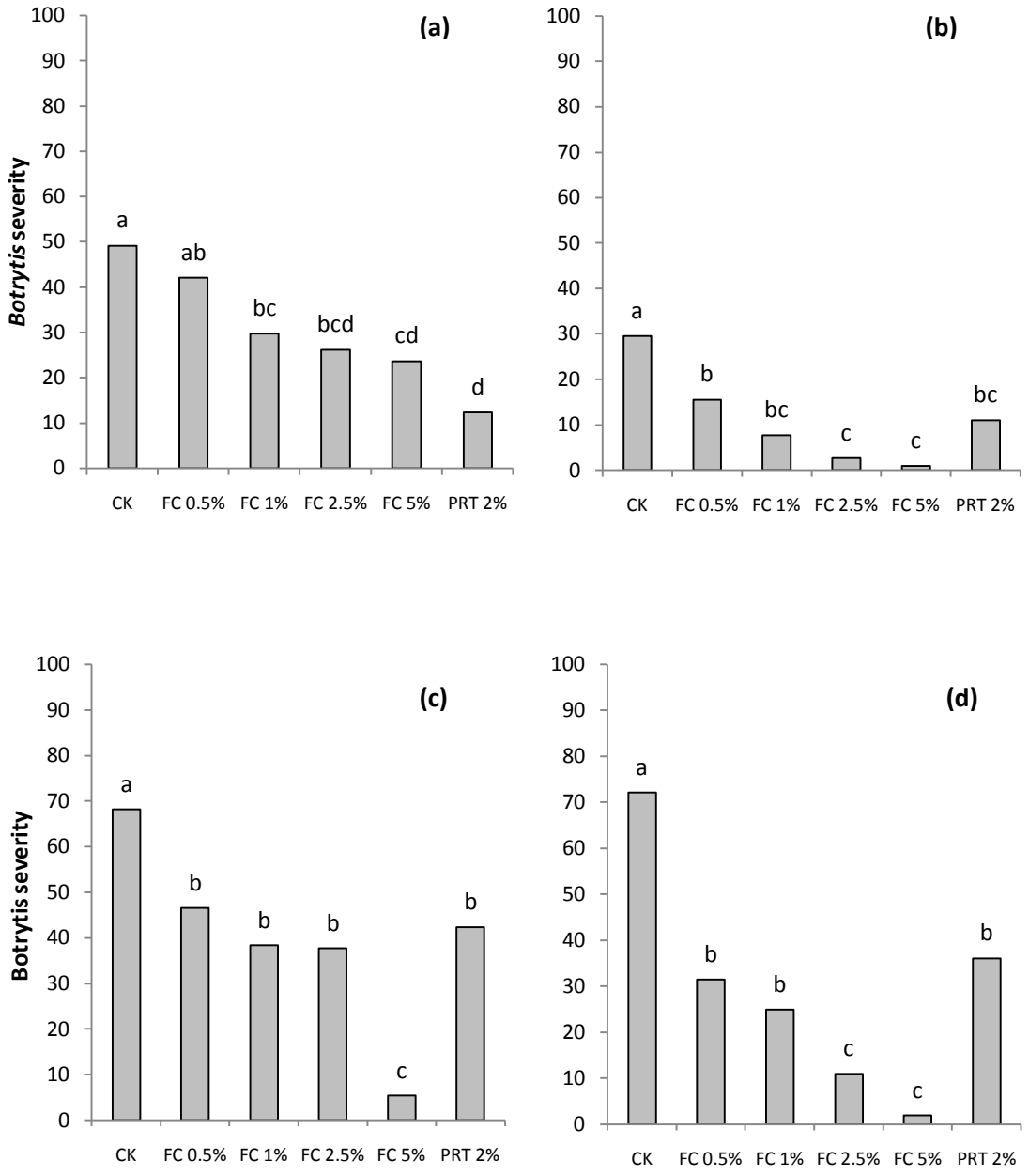


Figure 4 Effect of fatty acid based product Foodcoat (FC) on *B. cinerea* rot severity caused by inoculation of sound grape berries inoculated with mycelial plugs, after seven days of incubation at 22 ± 1 ºC. Chardonnay and Sauvignon berries were inoculated with 5 mm plugs of seven-day old cultures of Iso2 (Fig. 4a,c) and Iso7 (Fig. 4b,d), respectively. Rot severity was scored as proportion of grape berry surface with typical *B. cinerea* berry rot symptoms. Values are means of six replicates per isolate and cultivar. Mean values with the same letter are not significantly different ($P < 0{\cdot}05$) according to LSD test.

Effect of Foodcoat on B. cinerea *infection of wounded grape berries*

Statistical analysis showed there were not significant interactions ($P < 0{\cdot}05$) between variety, isolate and treatments. Therefore, data were pooled and summarized in Fig. 5.

All treatments significantly reduced ($P < 0{\cdot}05$) *B. cinerea* rot severity when berries were artificially wounded and inoculated with a suspension of *B. cinerea* conidia.

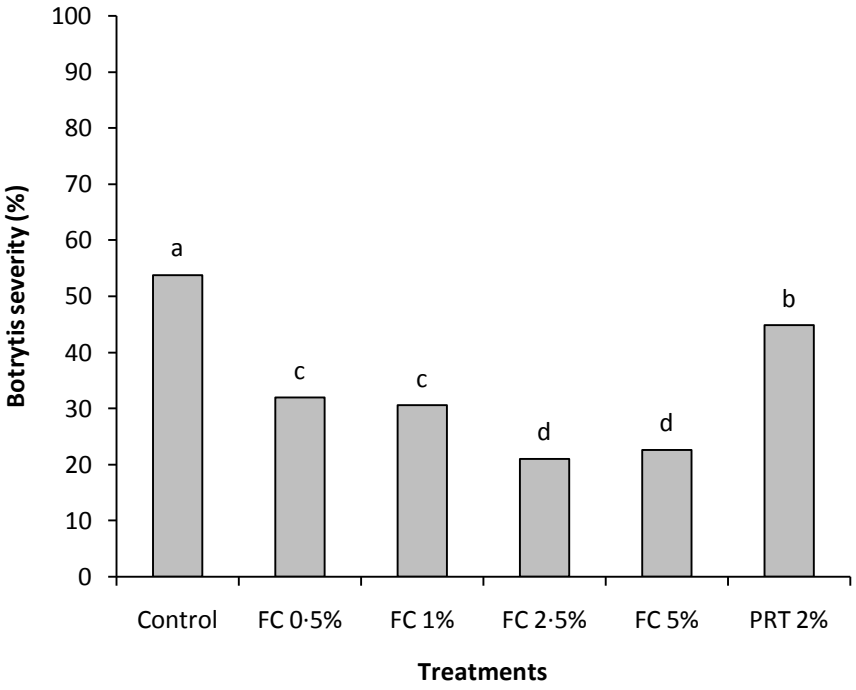


Figure 5 Effect of fatty acid based product Foodcoat (FC) on *B. cinerea* rot severity caused by conidial infection of wounded grape berries, after seven days of incubation at 22 ± 1 ºC. Chardonnay and Sauvignon blanc berries were wounded with fine grade sandpaper and sprayed with different concentrations of Foodcoat® (FC; 0·5, 1, 2·5, and 5 % v v^{-1}) and Protector^{HML} (PRT; 2 % v v^{-1}). Then, treated berries were inoculated with *B. cinerea* spore suspensions ($5x10^4$ conidia ml^{-1}) of isolates Iso2 and Iso7. Rot severity was scored as proportion of grape berry surface with typical *B. cinerea* berry rot symptoms. Values are means of six replicates per isolate and cultivar. Mean values with the same letter are not significantly different ($P < 0·05$) according to LSD test.

There was evidence of a dose response within the FC treatments since the high concentrations of FC (FC 2·5% and FC 5%) were significantly ($P < 0·05$) more effective at reducing *B. cinerea* severity, compared with lower FC concentrations (FC 0·5% and FC 1%). The FC 2·5% and FC5% treatments reduced *B. cinerea* severity by 60% and 57%, respectively. The PRT 2% treatment reduced *B. cinerea* severity by 16%, compared to the untreated control. Nonetheless, *B. cinerea* severity in the PRT 2% treatment (severity= 45%) was significantly higher ($P < 0·05$) than in any of the FC treatments, including the lower FC concentrations.

Contrary to the results observed after mycelial plugs inoculations, wounded berries cv. Chardonnay presented significantly higher overall severity ($P < 0·05$) than those cv. Sauvignon blanc, seven days after inoculation with conidial suspensions (42·5 and 25·7, respectively).

Effect of Foodcoat on berry fungal microbiota

The effect of FC applied to Sauvignon blanc grape bunches in a field trial (Marlborough, NZ) on the recoverable grape berry fungal microbiota is summarized in Fig. 6. Three applications of FC between veraison and harvest, regardless of FC concentration, significantly increased ($P < 0·05$) the number of pink and white yeasts on the grape berry surface, compared to the untreated control. The fungi and yeast-like fungi populations were unaffected by FC treatment compared to the untreated control.

White yeast populations increased significantly ($P < 0{\cdot}05$) by approximately 0·3 log units while the pink yeast populations increased significantly ($P < 0{\cdot}05$) by 0·7, 0·6 and 0·4 log units after treatment with FC 0·5, FC 1 and FC 2·5% respectively. The pink yeast CFU g^{-1} of grape berry recovered from samples treated with the higher dose of FC (FC 2·5%) was significantly lower ($P < 0{\cdot}05$), compared with FC at 0·5% suggesting that lower FC concentrations may be more beneficial to pink yeasts compared to higher FC concentrations.

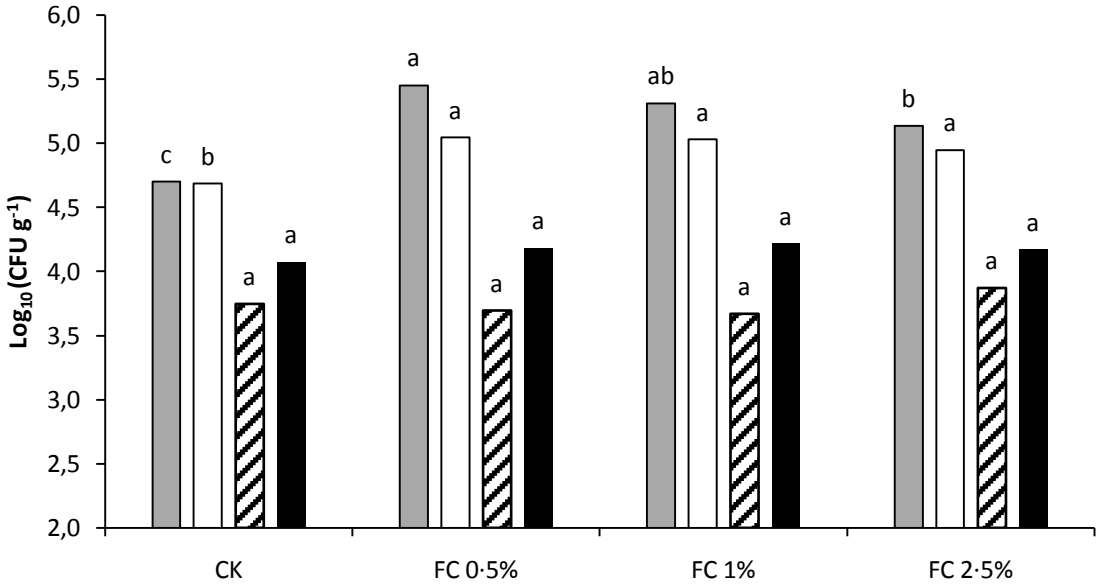


Figure 6 Effect of fatty acid based product Foodcoat (FC) field applications on fungal populations on Sauvignon blanc grape berries at harvest (Marlborough, NZ, 2011). Treated grape vines received three applications of different concentrations of Foodcoat (FC; 0·5, 1 and 2·5 % v v^{-1}) between veraison and preharvest, or no treatment (CK). After population recovery, counts of berry surface pink yeasts ( ), white yeasts ( ), yeast-like fungi ( ) and filamentous fungi ( ) were carried out based upon colony morphology on semi selective yeast agar or Rose Bengal Agar. Colony counts (CFU g^{-1}) were log transformed prior to ANOVA. Presented data are mean of six replicates. Mean values linked by the same letter for each fungal group are not significantly different ($P < 0{\cdot}05$) according to Student's *t* test.

Discussion

Previous research identified that FC was a beneficial adjuvant for *C. sake* CPA-1 significantly increasing field survival of this BCA, demonstrating some efficacy against BBR when used alone (Cañamás *et al.* 2011; Calvo-Garrido *et al.* 2013). These findings stimulated the research on the mechanisms by which *B. cinerea* control was achieved by fatty acid-based products, such as FC and PRT.

High FC concentrations (FC 2·5% and FC 5%) significantly reduced conidial germination by 29% and 65%, respectively, although this did not reach the levels of germination inhibition typical of most synthetic fungicides (Rosslenbroich and Stuebler 2000). The inhibition of germination of *B. cinerea* and other fungi in experiments with lauric, palmitic or estearic acids, the principle fatty acid components in the FC formulation, were also reported to be higher when applied alone (Alzaemey *et al.* 1993; Hou and Forman 2000; Rihakova *et al.* 2001; Walters

et al. 2003; Malcolm and Guzman 2007; Liu *et al.* 2008; Karlova *et al.* 2010). The PRT 2% treatment reduced germination in the present study, but also without reaching high germination inhibition. This suggests that the formulations used are not primarily fungitoxic and hence inhibition of conidial germination is not the FC or PRT main mode of action. A recent study published on the PRT website (http://www.henrymanufacturing.co.nz/products/protectorhml/bioassay-botrytis.pdf) indicated high germination inhibition rates by PRT 1% treatments. However, the assay was carried out with different *B. cinerea* isolates and without PDA medium (only SDW). This low nutrient availability and isolate sensitivity may have accounted for differences in germination inhibition between both studies.

The microscopic examination of *B. cinerea* germinated conidia exposed to FC treatments demonstrated that FC substantially altered morphology of conidia and germ tubes and this effect was linked to the FC concentration. The swollen and distorted germ tubes described in the results section, suggested significant metabolic alterations, an observation that has been reported in other studies evaluating the effect of synthetic fungicides, NPs or BCAs on fruit pathogens (Jijakli and Lepoivre 1998; Rosslenbroich and Stuebler 2000; Bryk *et al.* 2004; Reglinski *et al.* 2010), although this effect is reported for first time in this study for fatty acid-based NPs. Changes in cell morphology may also be due to the characteristics of the viscose FC matrix formed around *B. cinerea* conidia, which may interfere with nutrient uptake and water relations in the cell and may also restrict the enlargement of *B. cinerea* germ tubes, since the effects on germ tube morphology were more evident at the higher FC concentrations.

Overall, all FC treatments (FC 0·5% to FC 5%) significantly reduced infection of grape tissues by selected virulent *B. cinerea* strains. However, only in the berry infection experiments there was clear evidence of a dose-response effect, which was generally consistent for each cultivar, isolate and inoculation method used. The high FC concentrations (2·5% and 5%) were the most effective treatments and differences between these concentrations were significant only in the Sauvignon blanc berry assay that was inoculated with mycelial plugs of *B. cinerea* Iso2. Based on these findings that the high 5% rate did not contribute to improve *B. cinerea* control, the application of the 2·5% rate of FC can be considered as more cost effective to prevent *B. cinerea* infection of grape berries. Generally, the efficacy of FC and PRT was similar on unwounded berries. In contrast, the efficacy of the PRT 2% treatment on wounded berries inoculated with conidial suspensions was much less, compared with FC at 2·5% (17% and 61% reduction compared to control, respectively). These findings would suggest FC as the preferred product for *B. cinerea* control. However, since PRT has a lower market price, a careful cost analysis should be carried out before field applications. Different strategies may be also explored to reduce treatment cost, such as focused applications on key epidemiological stages, or the combination of fatty acid-based NPs with BCAs or other NPs, including the application of FC and PRT together.

Recently, a less concentrated formulation of FC (Fungicover® Base) achieved similar reductions of *B. cinerea* in field applications (Calvo-Garrido *et al.* 2013) and laboratory experiments (Calvo-Garrido et al., *unpublished*) and, to our knowledge, the present research and the client reports on PRT published by Henry Manufacturing Ltd (http://www.henrymanufacturing.co.nz/products/protectorhml/) are the only studies showing efficacy of fatty acid-based NPs against *B. cinerea* of grapes in the field and in laboratory studies. Nonetheless, other fatty acid-based

products effectively controlled *B. cinerea* in other fruit crops, such as ECO-100 SC applied postharvest on apples (Montealegre *et al.* 2010), or other experimental fatty acid formulation on cucumber (Hou and Forman 2000). Moreover, reports of the efficacy of fatty acid products against other plant pathogens are also found in bibliography. Al Zaemey *et al*. (1993) reported significant reductions of *Colletotrichum musae* on bananas by two commercialized coating materials (Semperfresh® and Semperfresh® acid-stable) based upon sucrose esters of fatty acids, including palmitic and lauric acids. The product Tecnobiol®, a formulation containing linoleic and oleic acids, showed moderate efficacy against powdery mildew of grapes caused by *Plasmopora viticola* (Palla 2006), whereas it was ineffective at reducing late blight of tomatoes caused by *Phytophthora infestans* (Ferrari *et al.* 2007). A similar formulation to PRT, based on potassium salts of fatty acids, did not give acceptable control of grapevine downy mildew caused by *Plasmopara viticola* (Vercesi *et al.* 2001). Moreover, non commercial formulations of lauric acid reduced barley powdery mildew (Walters *et al.* 2003) and combinations of palmitic and oleic acids reduced plant losses due to soil phytopathogenic fungi (Liu *et al.* 2008). Thus, the *B. cinerea* severity reduction rates by FC and PRT observed in the present study were similar or higher than those observed in the published research mentioned above, highlighting the potential of these two products compared to other fatty acid-based NPs against their target pathogens.

Investigation of the effect of FC on *B. cinerea* infection of grape tissues provided new data in order to more clearly define the mode of action of FC against BBR. When grape berries were unwounded, Chardonnay berries presented lower *B. cinerea* severity than Sauvignon blanc, whereas severity on Chardonnay berries was significantly higher when skin was wounded and berry biophysical barriers were substantially reduced. Since cv. Chardonnay is considered to have thicker skin than cv. Sauvignon blanc (Fuster 2006), differences in cultivar sensitivity may be related to berry skin protection of infection. In contrast, when the high doses of FC (2.5% and 5%) were applied, none of the differences in cultivar sensitivity or isolate aggressiveness were significant under any of the conditions tested. These findings on FC dose effect related to cultivar skin thickness suggest that the FC film on green tissues provides an increased physical barrier to *B. cinerea* infection, in addition to that provided by berry cuticle. Likewise, high efficacy and the clear dose effect of FC treatments protecting wounded berries would support this hypothesis. The enhancement of this berry natural defense may represent an important FC mechanism preventing *B. cinerea* infection.

In addition, FC significantly increased the natural berry surface white and pink yeast populations, which were very high also in the untreated samples, although they were similar to those observed in other studies (Barata *et al.* 2008; Barata *et al.* 2012). Since epiphytic microbiota on grape berries has a diverse range of bacteria, fungi and yeasts some of which are antagonistic to *B. cinerea* (Nally *et al.* 2012; Csutak *et al.* 2013), this effect may influence *B. cinerea* infection reduction via increased nutrient and space competition, thereby adding another further mechanism for FC efficacy in the field. Additionally, the efficacy of the FC field applications carried out in the microbiota study was also measured at harvest time. Nonetheless, incidence and severity of BBR at harvest was too low for the results to be meaningful and there were not significant differences. The addition of selected nutrient sources to enhance natural berry microbiota or BCA populations, thereby providing improved disease control, is called

'restorative biological control' and is regarded as an important control strategy for future research (Everett *et al.* 2005). Similarly, this finding on FC mode of action needs further investigations in order to confirm the *B. cinerea* suppression by these yeasts, as well as define the significant inverse dose effect observed in this study. Nonetheless, the findings reported in this investigation are the first that demonstrates the potentially positive effects of fatty acid NP applications on berry microbiota populations. The positive effect on berry microbiota supports the observations of Cañamás *et al.* (2011) that indicated elevated field survival of the yeast antagonist *C. sake* CPA-1 when applied with Fungicover, suggesting that the film coat created by this product generates a beneficial environment for the yeast BCA, thereby reducing cell mortality. One of the greatest advantages of these fatty acid-based NPs is their compatibility with BCAs, as described for *C. sake* CPA-1 with Fungicover or the fungal BCA, *U. oudemansii* with PRT. Therefore, although their efficacy alone may not be high enough to compete with synthetic fungicides, these combinations are especially attractive as strategies to improve the consistency of biologically based treatments in the field, improving their uptake by vineyard managers and the further reduction of synthetic fungicides for *B. cinerea* control in grapes.

Based on our findings and other studies, it is proposed that FC has multiple modes of action when applied in the field against *B. cinerea* through the establishment of a film coating on grapevine tissues. This film reduces spore germination and or debilitates germinated conidia interfering with *B. cinerea* conidial infection, whereas the FC barrier is also capable of blocking challenging mycelial infections and protecting wounds. It is also proposed that FC has an indirect effect on *B. cinerea* infection by increasing the naturally occurring yeast populations on the grape berry microbiota, thereby reducing the probability of infection. The precise mechanism by which yeast populations are significantly stimulated is not fully determined and requires further investigation under a range of field conditions and varieties of grapes. The results from this investigation also indicated that PRT had a different mode of action compared to FC. The PRT film coating may provide a poor environment for *B. cinerea* germination and infection, but without disrupting *B. cinerea* cell function. In addition interfered with the infection of green tissues although was insufficient to prevent *B. cinerea* infection of wounded berries. Complex interference by FC and PRT in the specific biochemical host-pathogen interaction prior to infection may also occur and requires further investigation. Other authors have associated the efficacy of fatty acid NPs with other indirect mechanisms such as a reduction in water availability (Alzaemey *et al.* 1993), or evaluated plant defense activation (Montealegre *et al.* 2010).

In conclusion, the fatty acid-based product FC has demonstrated consistent efficacy against *B. cinerea* and our results suggested that it has a multiple mode of action. The observed efficacy was very high (up to 96% compared to the untreated control on unwounded Chardonnay berries) and is similar to, or higher than that of other fatty acid-based NPs. The FC mode of action represents an interesting mechanism to improve plant natural physical defenses and increase competition for the *B. cinerea* ecological niche on the grape berry. These findings strongly suggest that FC and PRT would be valuable additives for combining with compatible BCAs and NPs.

Acknowledgements

Authors are grateful to the University of Lleida for the PhD grant to Carlos Calvo-Garrido and the Catalan Government for the overseas travel grant to encourage international research collaboration with Plant & Food Research Institute, Ruakura Research Centre, Hamilton, New Zealand. The authors also wish to thank General Manager of BioProtection, PFR, Ms Philippa Stevens for supporting this joint project and to Drs Tony Reglinski, Stephen Hoyte and Kirstin Wurms in the Biological Control and Natural Products Team of PFR for their valuable assistance. Thanks are also expressed to Dion Mundy and Victoria Raw for valuable technical support and assistance with treatment applications and sampling of research plots in Marlborough, New Zealand. We thank to Henry Manufacturing Ltd and Domca S.L. for the supply of PRT and FC, respectively.

References

Alzaemey, A.B., Magan, N. and Thompson, A.K. (1993) Studies on the effect of fruit coating polymers and organic acids on growth of *Colletotrichum musae* in vitro and on postharvest control of anthracnose of bananas. *Mycol Res* **97**, 1463-1468.

Bajpai, V.K., Kim, H., Hou, C.T. and Kang, S. (2009) Bioconverted products of essential fatty acids as potential antimicrobial agents. *New Biotechnol* **26**, 122-130.

Barata, A., Malfeito-Ferreira, M. and Loureiro, V. (2012) The microbial ecology of wine grape berries. *Int J Food Microbiol* **153**, 243-259.

Barata, A., Seborro, F., Belloch, C., Malfeito-Ferreira, M. and Loureiro, V. (2008) Ascomycetous yeast species recovered from grapes damaged by honeydew and sour rot. *J Appl Microbiol* **104**, 1182-1191.

Bryk, H., Dyki, B. and Sobiczewski, P. (2004) Inhibitory effect of *Pseudomonas* spp. on the development of *Botrytis cinerea* and *Penicillium expansum*. *Plant Prot Sci* **40**, 128-134.

Calvo-Garrido, C., Elmer, P.A.G., Viñas, I., Usall, J., Bartra, E. and Teixidó, N. (2013) Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1. *Plant Pathol* **62**, 510-519.

Calvo-Garrido, C., Viñas, I., Elmer, P.A., Usall, J. and Teixidó, N. (in press) Suppression of *Botrytis cinerea* on necrotic grapevine tissues by early-season applications of natural products and biological control agents. *Pest Man Sci*.

Cañamás, T.P., Viñas, I., Torres, R., Usall, J., Solsona, C. and Teixidó, N. (2011) Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. *Biol Control* **56**, 150-158.

Coleman, R. and Penner, D. (2008) Organic acid enhancement of pelargonic acid. *Weed Technol* **22**, 38-41.

Csutak, O., Vassu, T., Sarbu, I., Stoica, I. and Cornea, P. (2013) Antagonistic activity of three newly isolated yeast strains from the surface of fruits. *Food Technol Biotech* **51**, 70-77.

Cus, F., Cesnik, H.B., Bolta, S.V. and Gregorcic, A. (2010) Pesticide residues and microbiological quality of bottled wines. *Food Control* **21**, 150-154.

Desbois, A.P. (2012) Potential applications of antimicrobial fatty acids in medicine, agriculture and other industries. *Recent Pat Antiinfect Drug Discov* **7**, 111-122.

Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. (2004) *Botrytis* spp. and diseases they cause in agricultural systems - an introduction. In *Botrytis: biology, pathology and control* eds. Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. pp.1-8. Dordrecht, the Netherlands: Kuwer academic publishers.

Elmer, P.A.G. and Michailides, T.J. (2004) Epidemiology of *Botrytis cinerea* in orchard and vine crops. In *Botrytis: Biology, Pathology and control* eds. Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. pp.243-272. Dordrecht, the Netherlands: Kuwer academic publishers.

Elmer, P.A.G. and Reglinski, T. (2006) Biosuppression of *Botrytis cinerea* in grapes. *Plant Pathol* **55**, 155-177.

Everett, K.R., Vanneste, J.L., Hallett, I.C. and Walter, M. (2005) Ecological alternatives for disease management of fruit rot pathogens. *N Z Plant Protect* **58**, 55-61.

Ferrari, A., Dubeshko, S., Vintel, H., David, D.R. and Elad, Y. (2007) Integration of biocontrol agents and natural products against tomato late blight. *Bull OILB/SROP* **30**, 437-440.

Fuster, P.M.C. (2006) *Variedades de vid: registro de variedades comerciales. 2 ed*. Madrid: Ministerio de Agricultura, Pesca y Alimentación.

Hou, C.T. (2008) New bioactive fatty acids. *Asia Pac J Clin Nutr* **17**, 192-195.

Hou, C.T. and Forman, R.J. (2000) Growth inhibition of plant pathogenic fungi by hydroxy fatty acids. *J Ind Microbiol Biot* **24**, 275-276.

Jijakli, M.H. and Lepoivre, P. (1998) Characterization of an exo-beta-1,3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. *Phytopathology* **88**, 335-343.

Karlova, T., Polakova, L., Smidrkal, J. and Filip, V. (2010) Antimicrobial effects of fatty acid fructose esters. *Czech J Food Sci* **28**, 146-149.

Komárek, M., Čadková, E., Chrastný, V., Bordas, F. and Bollinger, J.C. (2010) Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. *Environ Int* **36**, 138-151.

Liu, S., Ruan, W., Li, J., Xu, H., Wang, J., Gao, Y. and Wang, J. (2008) Biological control of phytopathogenic fungi by fatty acids. *Mycopathologia* **166**, 93-102.

Ma, C. and Gu, Z. (2003) Insecticidal activity of fatty acid compounds and their potential in controlling pests of cabbage in the field. *Acta Agr Shanghai* **19**, 101-104.

Malcolm, P. and Guzman, M. (2007) In vitro activity of fatty acids and natural fungicides on *Fusarium* sp. and *Colletotrichum musae*, causal agents of post-harvest diseases in bananas (Musa AAA). *Corbana* **33**, 15-27.

Montealegre, J.R., Lopez, C., Stadnik, M.J., Henriquez, J.L., Herrera, R., Polanco, R., Di Piero, R.M. and Perez, L.M. (2010) Control of grey rot of apple fruits by biologically active natural products. *Trop Plant Pathol* **35**, 271-276.

Nally, M.C., Pesce, V.M., Maturano, Y.P., Munoz, C.J., Combina, M., Toro, M.E., Castellanos de Figueroa, L.I. and Vazquez, F. (2012) Biocontrol of *Botrytis cinerea* in table grapes by non-pathogenic indigenous *Saccharomyces cerevisiae* yeasts isolated from viticultural environments in Argentina. *Postharvest Biol Tec* **64**, 40-48.

Palla, O. (2006) A new formulation based on fatty acids coming from vegetal oils effective against grapevine downy mildew. In *Giornate Fitopatologiche 2006* eds. Brunelli, A., Canova, A. and Collina, M. pp.17-20. Riccione, Italia.

Penaflor, M., Victor, S.R., Bueno, O.C., Hebling, M.J.A., Pagnocca, F.C., Leite, A.C., Fernandes, J.B., Vieira, P.C. and da Silva, M. (2006) Toxicity of straight-chain fatty acids to leaf-cutting ants *Atta sexdens rubropilosa* (Hymenoptera : Formicidae) and the symbiotic fungus *Leucoagaricus gongylophorus*. *Sociobiology* **47**, 843-858.

Reglinski, T., Elmer, P.A.G., Taylor, J.T., Wood, P.N. and Hoyte, S.M. (2010) Inhibition of *Botrytis cinerea* growth and suppression of botrytis bunch rot in grapes using chitosan. *Plant Pathol* **59**, 882-890.

Ricke, S.C., Kundinger, M.M., Miller, D.R. and Keeton, J.T. (2005) Alternatives to antibiotics: Chemical and physical antimicrobial interventions and foodborne pathogen response. *Poultry Sci* **84**, 667-675.

Rihakova, Z., Plockova, M., Filip, V. and Smidrkal, J. (2001) Antifungal activity of lauric acid derivatives against *Aspergillus niger*. *Eur Food Res Technol* **213**, 488-490.

Romanazzi, G., Lichter, A., Gabler, F.M. and Smilanick, J.L. (2012) Recent advances on the use of natural and safe alternatives to conventional methods to control postharvest gray mold of table grapes. *Postharvest Biol Tec* **63**, 141-147.

Rosslenbroich, H.J. and Stuebler, D. (2000) *Botrytis cinerea* - history of chemical control and novel fungicides for its management. *Crop Prot* **19**, 557-561.

Ruiz-Rodriguez, A., Reglero, G. and Ibañez, E. (2010) Recent trends in the advanced analysis of bioactive fatty acids. *J Pharmaceut Biomed* **51**, 305-326.

Tardaguila, J., Blanco, J.A., Poni, S. and Diago, P. (2012) Mechanical yield regulation in winegrapes: comparison of early defoliation and crop thinning. *Aust J Grape Wine R* **18**, 344-352.

Tripathi, P. and Dubey, N.K. (2004) Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biol Tec* **32**, 235-245.

Vercesi, A., Pontiroli, R. and Rizzotti, R. (2001) Organic viticulture and control. *Vignevini* **28**, 55-62.

Walker, A.-S., Micoud, A., Remuson, F., Grosman, J., Gredt, M. and Leroux, P. (in press) French vineyards provide information that opens ways for effective resistance management of *Botrytis cinerea* (grey mould). *Pest Man Sci* **69**, 667-678.

Walters, D., Raynor, L., Mitchell, A., Walker, R. and Walker, K. (2004) Antifungal activities of four fatty acids against plant pathogenic fungi. *Mycopathologia* **157**, 87-90.

Walters, D.R., Walker, R.L. and Walker, K.C. (2003) Lauric acid exhibits antifungal activity against plant pathogenic fungi. *J Phytopathol* **151**, 228-230.

Wang, C.L., Xing, J.S., Chin, C.K. and Peters, J.S. (2002) Fatty acids with certain structural characteristics are potent inhibitors of germination and inducers of cell death of powdery mildew spores. *Physiol Mol Plant P* **61**, 151-161.

Wurms, K., Chee, A., Elmer, P., Agnew, R., Wood, P. and Chee, A.A. (2011) Developing new biologically-based products for control of botrytis bunch rot. Part 1: Developing a new natural product for mid-season botrytis control - NP2 moves closer to the market. *Wine Vitic J* **26**, 64-72.

Zhao, H., Kim, Y.K., Huang, L. and Xiao, C.L. (2010) Resistance to thiabendazole and baseline sensitivity to fludioxonil and pyrimethanil in *Botrytis cinerea* populations from apple and pear in Washington State. *Postharvest Biol Tec* **56**, 12-18.

CAPÍTULO 5

Potential secondary inoculum sources of *Botrytis cinerea* and their influence on bunch rot development in dry Mediterranean climate vineyards

C. Calvo-Garrido, J. Usall, I. Viñas, P.A.G. Elmer, E. Cases and N. Teixidó

Pest Management Science **(en prensa), DOI 10.1002/ps.3629**

Research Article

Received: 16 May 2013 Revised: 17 July 2013 Accepted article published: 20 August 2013 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.3629

Potential secondary inoculum sources of *Botrytis cinerea* and their influence on bunch rot development in dry Mediterranean climate vineyards

Carlos Calvo-Garrido,[a] Josep Usall,[b] Inmaculada Viñas,[a] Philip AG Elmer,[c] Elena Cases[b] and Neus Teixidó [b]*

Abstract

BACKGROUND: Epidemiological studies have described the life cycle of *B. cinerea* in vineyards. However, there is a lack of information on the several infection pathways and the quantitative relationships between secondary inoculum and bunch rot at harvest.

RESULTS: Over two seasons, different spray programmes were used to determine key phenological stages for bunch rot development. Secondary inoculum sources within the bunch were also studied. The relative importance of flowering was evidenced in the given conditions, as treatments that included two fungicide applications at flowering were the most effective. In 2010, under conducive meteorological conditions for *B. cinerea* development after veraison, an extra application provided significantly higher control. Infections of necrotic tissues inside the bunch and latent infections developed mainly during flowering, while very low quantities of *B. cinerea* conidia were recovered from the fruit surface at veraison. Regression analysis correlated the incidence of latent infections and *B. cinerea* incidence on calyptras and aborted fruits at veraison with incidence of *Botrytis* bunch rot at harvest, presenting $R^2 = 0.95$ for the overall regression model.

CONCLUSION: This work points out key phenological stages during the season for bunch rot and *B. cinerea* secondary inoculum development and relates quantitatively inoculum sources at veraison to bunch rot at harvest. Recommendations for field applications of antibotrytic products are also suggested.

Keywords: epidemiology; fungicide timing; latent infections; necrotic tissues; surface inoculum; regression

1 INTRODUCTION

Botrytis bunch rot (BBR) of grapevine is one of the most important diseases of this crop, producing losses in quality and quantity in both wine and table grape vineyards.[1] The causal agent is *Botrytis cinerea*, a necrotrophic pathogen affecting many different crops that is able to survive, grow and infect under a wide range of conditions.[2,3] It infects green parts of the plant as well as necrotic tissues present in the canopy, from pruning and plucked leaves to floral and fruit debris. *B. cinerea* also develops resistance structures to overwinter and, in addition, produces latent infections that remain asymptomatic, allowing itself to oversummer in host tissues until berries start to ripen at veraison.[4]

The described features of the pathogen's ecology result in a highly complex life cycle where host–pathogen–environment interactions are affected by vineyard and cultivar characteristics, host resistance features, previous cultural practices, plant health and nutrition, disease vectors, *B. cinerea* populations and meteorological conditions.[5] Interactions among these factors may change during the season and from one winemaking region to another. Thus, bunch rot control may rely on different strategies depending on each region and climatic conditions to overcome that variability.

Disease control has been conducted mainly through synthetic fungicide application on a regular timing basis or focusing fungicide applications on key stages during the season. However, *B. cinerea* has developed resistance to many of the antibotrytic active ingredients,[6,7] making chemical control ineffective in some cases. Moreover, public concern about the effect of synthetic fungicides on the environment and human health has provided an incentive for more restrictive regulations for its use,[8] establishing a maximum residue limit for grapes and wines produced within the European countries (EC Regulation No. 396/2005). Thus, there is a need for an improvement in fungicide control and the

* *Correspondence to: N Teixidó, IRTA, XaRTA-Postharvest, 191 Rovira Roure, 25198 Lleida, Catalonia, Spain. E-mail: neus.teixido@irta.cat*

a *Food Technology Department, Lleida University, XaRTA-Postharvest, Agrotecnio Center, Lleida, Catalonia, Spain*

b *IRTA, XaRTA-Postharvest, Lleida, Catalonia, Spain*

c *The New Zealand Institute for Plant and Food Research Limited, Ruakura Research Centre, Hamilton, New Zealand*

development of new control strategies based on reduced pesticide input and non-synthetic products such as biological control and natural products.

Deep knowledge of the *B. cinerea* disease cycle, especially regarding pathogen ecology on leaves and bunches, latent infections and the importance of *B. cinerea* inoculum, is regarded as an important basis for developing effective strategies.[5,9–12] Further investigations are required to gain a full understanding of infection pathways for *B. cinerea* in grapes, described by Elmer and Michailides,[5] such as latent infections (pathway II), necrotic tissues of the bunch (pathways III and IV) and *B. cinerea* inoculum on the fruit surface (pathway V). Recently, significant correlation between *B. cinerea* found in grapevine stamens and the percentage of infected berries has been reported.[9] Nonetheless, there are few published research works relating quantitative data on this and other secondary inoculum sources in the canopy to bunch rot at harvest.[13,14]

Field studies working with fungicide timing or artificial inoculations have delimited essential stages of the epidemic for different regions and cultivar characteristics.[15–17] This information, along with basic research data, has resulted in the development of more efficient applications of alternative strategies, reduced pesticide strategies and disease warning systems,[18–20] providing valuable tools for an effective and more sustainable disease control. However, most of this research has been done in cool temperate regions; there are few epidemiological studies in dry summer climate areas, such as the Mediterranean region, and no studies are reported from Spain.[21–23] In these regions, some aspects of BBR epidemiology may work similarly, while others may be different owing to climatic conditions, hence modifying disease control strategies.

Common application practices in Spain follow the 'standard' phenological method developed in the 1960s for French vineyards, which recommends four sprays between flowering and harvest.[24,25] This standard, however, is based on a very conducive climate scenario for *B. cinerea* development, different from most of the winegrowing regions in Spain, where applications are frequently reduced depending on the practical experience of growers or recommendations of the governmental extension service. Moreover, there is no specific BBR risk prediction model for dry Mediterranean conditions, although few validations or adaptations of other predictive models are available in Spain.[26,27]

BBR in the Mediterranean region is not as destructive as it can be in cooler and more humid regions. Nonetheless, Spain is the third largest wine producer worldwide, with more than 3 339 700 t year^{-1} produced on 1 002 210 ha,[28,29] and bunch rot can also be a major problem depending on vineyard location and meteorological conditions during the season. Therefore, specific epidemiological research in the region provides helpful information to many growers in Spain, but also in other regions with similar climatic conditions.

In this study, field and laboratory-based experiments were conducted over two seasons in different vineyard locations in the Lleida area, Catalonia, Spain. The main objective was to identify the key moments to apply antibotrytic treatments and achieve effective disease control, and hence to define the most important phenological stages in the growing season for BBR development. In addition, inoculum sources in the bunch were studied in order to describe the relative importance of these sources in the present system and ultimately to correlate inoculum data with bunch rot at harvest.

2 MATERIALS AND METHODS

2.1 Vineyard location and characteristics

Two commercial vineyards were used for the fungicide timing experiment in 2009, while only one of these vineyards was used for all the 2010 experiments. A third field site was used in the 2010 season to collect samples from untreated bunches (four replicate units), which were included only in the quantitative analysis of the relationship between secondary inoculum and bunch rot at harvest. The three vineyard sites are included in the designation of origin Costers del Segre, a traditional winemaking region which comprises a broad part of the Lleida area, Catalonia, Spain, and presents an inland, dry, Mediterranean climate with arid summer season characteristics. The 30 year meteorological data series of the area indicates that accumulative rainfall over the year is 428.4 mm, the mean maximum temperature is 30.2 °C between June and September and the average thermal amplitude between the warmest and coldest month is very high (21 °C).[30] Macabeu was the *Vitis vinifera* L. cultivar used for experiments owing to its high susceptibility to fungal rots, favoured by its compact bunch structure. Vineyard training was in the traditional bush system employed in the region.

2.2 Fungicide timing experiments

Six key phenological stages, from early flowering to harvest, were selected: 1–5% flowering; 80% flowering; pre-bunch closure; veraison; 21 days before harvest; 7 days before harvest. Then, 0.8 mL L^{-1} of iprodione 50% (w/v) (Sipcam, Spain), an antibotrytic contact fungicide with reduced persistence in plant tissues ($t_{1/2}$ = 3–7 days in the field),[31] was applied following the application timetable described in Table 1, spraying developing bunches or flowers until run-off. At harvest, BBR was evaluated in the treated plots and the untreated control.

Only one fungicide application was carried out at each phenological stage, whereas the six phenological stages were grouped in four season stages for an easier interpretation of results: (1) early season (Early), from the beginning to the end of flowering; (2) mid-season (Mid), from the end of flowering to the start of veraison; (3) late season (Late), from veraison to the last week before harvest; (4) 7 days before harvest (7 days Preharvest).

Treatments were sorted in a randomised block design with six replicates. Replicate plots consisted of a vine row of seven vines in which the five central vines were used for bunch rot assessment while the first and last were considered to be buffer vines.

Flowers and grape bunches were sprayed until run-off with a motorised backpack sprayer (WJR2225 model; Honda Motor Company Ltd, Germany) with a 1 mm nozzle and 15 bar pressure. A weather station (Decagon Services Inc., Pullman, WA) collected meteorological data during the growing season.

At harvest, BBR incidence (percentage of bunches with visual *B. cinerea* infection symptoms) and severity (percentage of *B. cinerea*-infected berries per bunch) were assessed on 50 bunches per replicate plot, selecting 25 bunches from each side of the vine row.

The same experiment was repeated in the 2010 season, using the experimental design and methodology described above, to increase the consistency of the results obtained.

2.3 Inoculum source quantification

In the 2010 season, *B. cinerea* inoculum sources within grape bunches were evaluated at veraison (before fungicide application at that stage) in order to analyse the relative contribution of the

Table 1. Treatment structure of the fungicide timing experiments (2009 and 2010 growing seasons)

	Early season		Mid season	Late season		
Treatment[a]	1–5% flowering	80% flowering	Pre-bunch closure	Veraison	21 days before harvest	7 days before harvest
Control	−	−	−	−	−	−
Early	+	+	−	−	−	−
Early + Mid	+	+	+	−	−	−
Early + Late	+	+	−	+	+	−
Mid + Late	−	−	+	+	+	−
Late	−	−	−	+	+	−
7 days Preharvest	−	−	−	−	−	+

[a] Treatments consisted of field applications (+) of antibotrytic fungicide iprodione at a dose of 0.8 mL L^{-1} at key phenological stages of the season.

different phenological stages to inoculum development prior to veraison. For this purpose, evaluation of the potential secondary inoculum was carried out over the six replicates of untreated vines (Control), vines treated during flowering (Early), vines treated at flowering and pre-bunch closure (Early + Mid) and vines treated at pre-bunch closure (Mid + Late). Potential inoculum sources studied in these plots were berry latent infections, necrotic tissues inside the bunch and berry surface inoculum.

In addition, the quantitative data of these secondary inoculum sources in untreated plots were used to correlate the different types of potential secondary inoculum with bunch rot incidence at harvest through regression analysis. In this analysis the authors evaluated data from the six untreated replicate plots of the fungicide timing experiment, plus data from four replicate units of the extra untreated field site.

2.4 Incidence of *B. cinerea* latent infections on grape berries

Six bunches were taken per replicate plot at veraison, selecting three bunches randomly from each side of the vine row. Then, five apparently sound berries were collected with sterile scissors from each bunch, leaving the pedicel attached, two from the top, two from the middle and one from the bunch bottom, resulting in a final sample of 30 grape berries per replicate plot and treatment. Berries were surface sterilised by dipping them (1) for 10 s in a 70% ethanol solution, then (2) for 4 min in a solution of NaOCl 0.5% (v/v) in sterile deionised water with Tween-80 (0.5 mL L^{-1}) and finally (3) for 2 min in sterile deionised water to rinse. Then, berries were placed onto sterilised plastic grids in plastic containers to let dry and, once dried, stored in a freezer at −20 °C overnight to terminate natural resistance of berries, induce tissue senescence and promote symptom development of the latent infections. After the freezing process, samples were incubated at 20 °C in high-humidity chambers for 15 days prior to result assessment. High-humidity chambers consisted of a plastic container and a plastic grid with a sterile filter paper moistened with 50 mL of sterile water between them, then introduced into plastic bags.

Percentages of incidence of latent infections (% Inc lat) were visually determined with the help of a Leica MZ16F stereoscope (Leica Microsystems GmbH, Germany), considering incidence as the presence of *B. cinerea* mycelium with at least one visible conidiophore. Percentage values resulting from the 30 berries per replicate were used for the subsequent statistical analysis.

2.5 *B. cinerea* incidence on necrotic tissues in developing bunches

At veraison, samples of necrotic tissues that were trapped within the developing bunches were taken from four bunches per replicate. Bunches were randomly selected from the sample vines, and in the laboratory each bunch was divided into four sections (top left, top right, middle and bottom of the bunch). Three different samples of necrotic tissues were collected with sterile tweezers from each bunch section, consisting of one aborted flower, one calyptra and one aborted fruit. Aborted fruits were considered to be different from aborted flowers when the swollen ovary of the transforming flower presented a diameter greater than 2 mm. Trays, gloves and tweezers were surface sterilised with ethanol prior to sectioning each bunch and removing necrotic samples.

The four samples of each tissue type from each bunch were placed on a sterile petri dish with Whatman No. 1 (85 mm diameter) filter paper that had been moistened with 1.25 mL of sterile distilled water to create a high-humidity chamber for *B. cinerea* development. The four high-humidity chambers corresponding to four bunches constituted one replicate. In total there were 16 aborted flowers, 16 calyptras and 16 aborted fruit per replicate. The petri dishes were then incubated at 20 °C in the dark for 10 days prior to assessment. Incidence of *B. cinerea* was visually assessed with a stereoscope for each tissue type as the presence of *B. cinerea* mycelium with at least one visible conidiophore. Mean values per replicate were calculated with the corresponding 16 processed samples per tissue type.

Resulting variables were the overall percentage incidence on necrotic tissues and the specific percentage incidence for each tissue type, namely: percentage incidence on aborted flowers (% Inc ab flowers), percentage incidence on aborted fruits (% Inc ab fruits) and percentage incidence on calyptras (% Inc calyptras).

2.6 Determination of *B. cinerea* inoculum on the berry surface

Similarly to the latent infection experiment, six bunches were randomly sampled from both sides of the replicate plot at veraison. Then, 30 berries with the pedicel attached were collected from each bunch with sterile scissors, and the corresponding 180 berries per replicate were weighed and placed into 200 mL of sterile water plus 0.5 mL L^{-1} of Tween-80. Submerged samples were then shaken for 30 min at 150 rpm in a rotary shaker (Selecta, Spain). After shaking, grape berries were removed, and the solution containing berry surface materials and microbiota, including *B. cinerea* conidia, was

centrifuged at 14 000 × *g* for 10 min at 10 ± 1 °C. Resulting pellets were resuspended in 1 mL of sterile deionised water plus Tween-80 (0.5 mL L^{-1}) and, after serial dilution, plated onto petri plates containing Edwards' *Botrytis* selective medium modified by increasing the agar concentration to 30 g L^{-1}.[32] After 7 days incubation in darkness at 20 °C, *B. cinerea* colonies were counted and referred to sample weight, and the results were expressed as colony-forming units per gram (CFU g^{-1}).

2.7 Statistical analysis

Treatment effects on BBR incidence and severity, incidence of latent infections, *B. cinerea* incidence in necrotic tissues and surface inoculum were analysed by analysis of variance. Significant treatment differences were determined by Student's least significant difference (LSD) *t*-test ($P = 0.05$) for all experiments. Data on surface inoculum, *B. cinerea* incidence on necrotic tissues and incidence of latent infections were transformed [square root ($x + 0.5$)] prior to ANOVA to minimise differences in variance among means. The relationships between explanatory variables and bunch rot incidence and severity at harvest were analysed by multiple linear regression. Variables were selected by the stepwise method with a $P = 0.05$ significance level to stay in the model. All data analysis was performed using JMP8 Statistical Discovery™ software (SAS Institute Inc., Cary, NC).

3 RESULTS

3.1 Fungicide timing experiments

Mean BBR severity exceeded 5% in all trials, indicating that treatment effects could be evaluated adequately. Incidence and severity of BBR at the two evaluated field sites in 2009 presented similar patterns, and interaction between treatment effects and field site was not significant ($P < 0.05$); therefore, data from the two fields were pooled and presented and analysed together (Fig. 1a). In the 2010 season, the trial was conducted only in one field and results are presented in Fig. 1b.

In the 2009 season, the untreated control presented 39.5% incidence and 5.5% severity, while higher levels of BBR incidence and severity were observed in the 2010 season, with 68.0% incidence and 12.6% severity. Overall, all treatments significantly reduced incidence and severity of BBR, except the 7 days Preharvest treatment in 2010.

The greatest reductions in BBR incidence and severity occurred with treatments involving fungicide applications at 5 and 80% flowering (Early, Early + Mid and Early + Late treatments). Therefore, additional applications at pre-bunch closure (Early + Mid) or veraison and 21 days preharvest (Early + Late) did not contribute to greater reductions in BBR incidence or severity. Only the Early + Mid treatment in 2010, when harvest incidence and severity were higher, presented significantly lower incidence than the Early treatment.

In contrast, the treatments consisting of fungicide applications from veraison to harvest (Mid + Late, Late and 7 days Preharvest) resulted in higher incidence and severity compared with treatments involving sprays during flowering. These treatments presented significantly higher incidence ($P < 0.05$) than the Early and Early + Mid treatments in 2009 (Fig. 1a), and higher than Early + Mid and Early + Late in 2010 (Fig. 1b).

The Late and 7 days Preharvest treatments presented significantly higher overall severity than the rest of the treatments in 2009, while no significant differences in severity were observed among effective treatments in 2010.

3.2 Inoculum source evaluation

The results of the evaluation of *B. cinerea* incidence on necrotic tissues within bunches are summarised in Fig. 2. The incidence of *B. cinerea* infections of necrotic tissues in untreated plots was 8.3%. Samples from plots sprayed during flowering (Early) and samples that had received an extra application at pre-bunch closure (Early + Mid) did not show *B. cinerea* infections. A single iprodione application at pre-bunch closure (Mid + Late) significantly reduced the *B. cinerea* incidence on necrotic tissues at veraison, presenting 4.0% incidence.

Latent infections on grape berries were observed on 4.4% of samples from the untreated control (Fig. 3). Two applications of iprodione at flowering (Early and Early + Mid treatments) significantly reduced incidence to 1% or less, whereas the addition of an application at pre-bunch closure did not yield a significant benefit compared with flowering sprays alone. One application at pre-bunch closure (Mid + Late treatment) was not able to reduce latent infections significantly.

No significant differences between treatments and the unsprayed control were observed in surface inoculum counts, which remained under 0.2 CFU g^{-1} in all cases (data not shown).

3.3 *B. cinerea* inoculum at veraison and BBR at harvest

Inoculum quantification of the different sources was studied in the untreated plots of the 2010 site, together with the additional data from the extra untreated field site.

Pearson's correlation coefficients (Table 2) and variance inflation factors (VIFs) (Table 3) were studied in order to detect multicollinearity within the set of explanatory variables. None of the variables presented high correlation coefficients, and the correlations observed were not significant ($P < 0.05$). Moreover, the VIF values of the explanatory variables were low (2.64 or less). Therefore, multicollinearity was unlikely to occur, and all the predictor variables were used for the subsequent regression analysis of BBR incidence and severity.

Multiple linear regression analysis (Table 3) included % Inc lat as the predominant explanatory variable in estimating BBR incidence ($R^2 = 0.49$). Two more variables were included in the model: % Inc calyptras and % Inc ab fruits. Both variables added 0.25 and 0.20, respectively, to the model's overall R^2, which was very high (0.95) and was also highly significant ($P = 0.0002$).

The severity of BBR was not significantly correlated with any of the explanatory variables analysed (data not shown).

3.4 Meteorological data

Meteorological conditions between 1–5% flowering and veraison presented slight variations when the two seasons were compared. The average temperature was 22.8 and 22.1 °C in 2009 and 2010 respectively, while the average relative humidity (RH) was 61.8% in both seasons, although rainfall in 2009 was higher than in 2010 (25.0 mm as opposed to 21.7 mm). Nonetheless, conditions after veraison were different when the two seasons were compared. The 2009 season was consistently dryer and warmer (63.4% RH, 22.7 °C, 3.0 mm rainfall) than the 2010 season (69.9% RH, 21.6 °C, 8.5 mm rainfall). Conditions of high humidity and average temperatures close to 20 °C registered between veraison and harvest in 2010 are considered to be favourable for BBR development.

4 DISCUSSION

The present study represents the first epidemiological study on BBR reported from Spain and illustrates some key aspects of *B. cinerea*

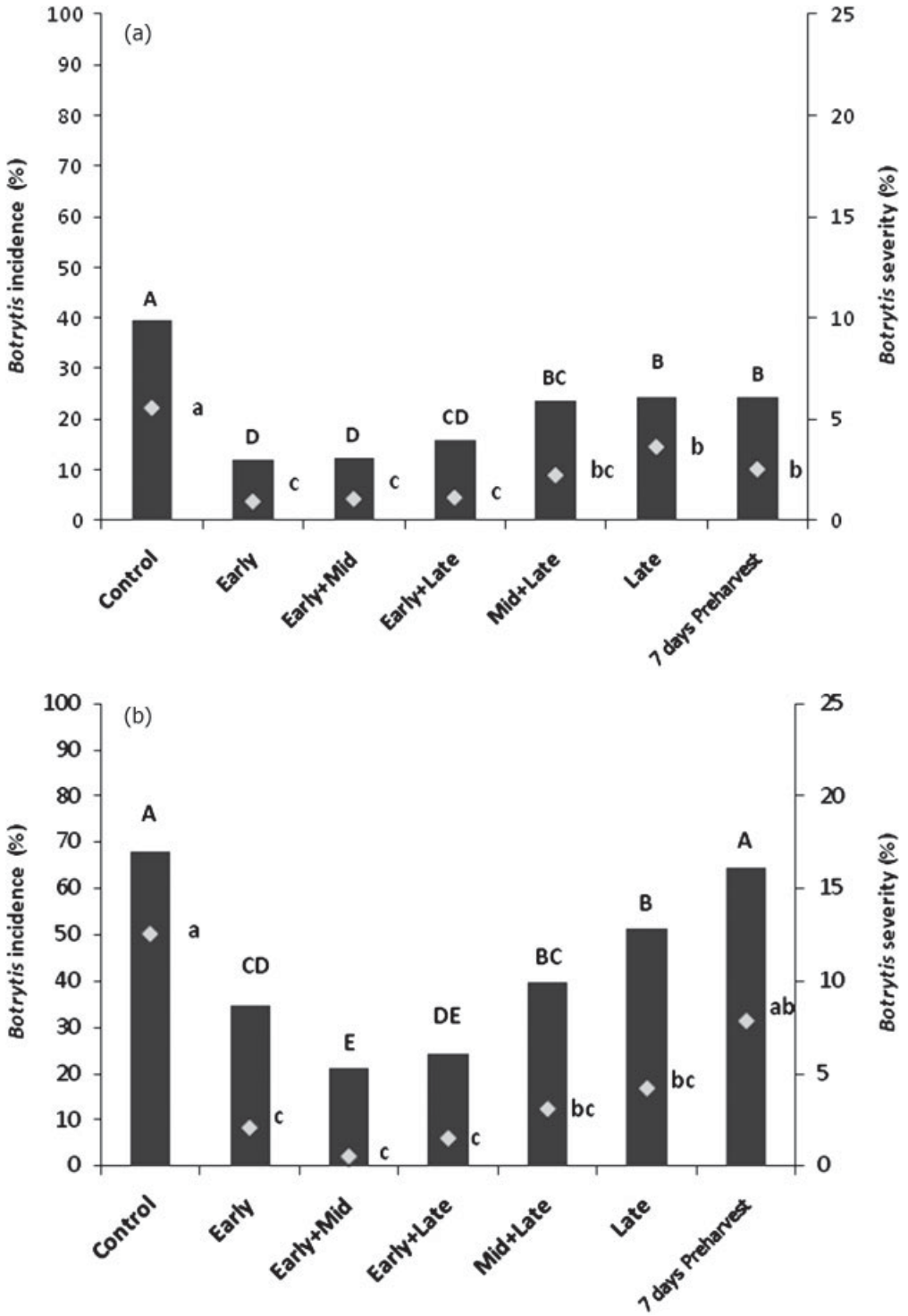


Figure 1. *Botrytis* bunch rot at harvest in Macabeu wine grapes. Incidence (bars) and severity (diamonds) were scored on 50 bunches per replicate plot in (a) two vineyards in 2009 and (b) one vineyard in 2010. Treatments consisted of field applications of the antibotrytic contact fungicide iprodione at a dose of 0.8 mL L^{-1} at key phenological stages. Control: untreated; Early: two applications at flowering; Early + Mid: two applications at flowering and one at pre-bunch closure; Early + Late: two applications at flowering and two applications from veraison to harvest; Mid + Late: one application at pre-bunch closure and two applications from veraison to harvest; Late: two applications from veraison to harvest; 7 days Preharvest: one application 7 days before harvest. Presented data are means of (a) twelve or (b) six replicates. Values linked by the same letter in the same case (upper case or lower case) are not significantly different ($P = 0.05$) according to Student's LSD *t*-test.

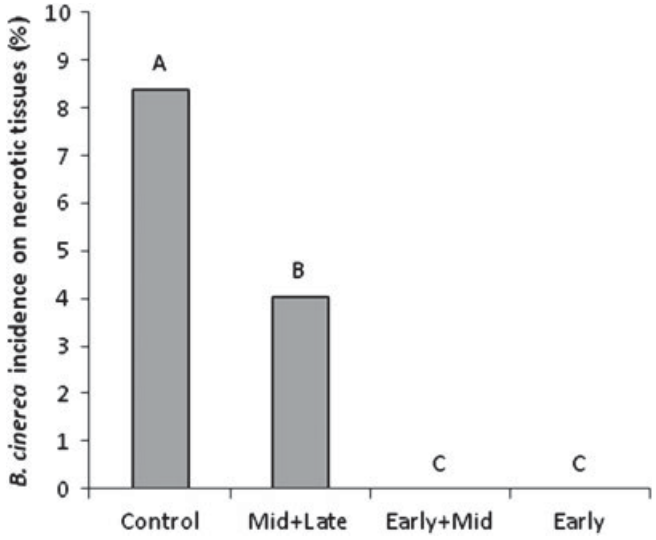


Figure 2. *Botrytis cinerea* incidence on necrotic tissues inside the bunch at veraison. Treatments consisted of field applications of antibotrytic fungicide (iprodione 0.8 mL L^{-1}) at key phenological stages of the season. Control: untreated; Mid + Late: one application at pre-bunch closure; Early + Mid: two applications at flowering and one at pre-bunch closure; Early: two applications at flowering. Incidence was scored as the observation of *B. cinerea* mycelium with at least one visible conidiophore. Presented values are means of six replicates. Mean values linked by the same letter are not significantly different ($P = 0.05$) according to Student's LSD *t*-test.

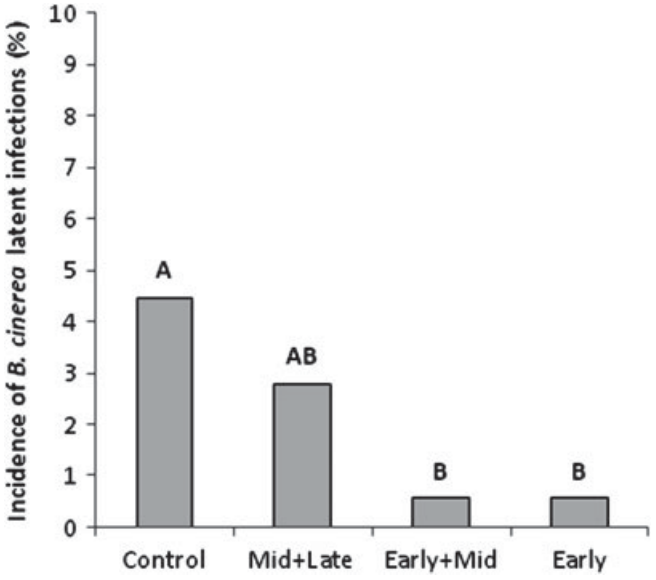


Figure 3. Incidence of latent infections in Macabeu grape berries at veraison. Treatments consisted of field applications of antibotrytic fungicide (iprodione 0.8 mL L^{-1}) at key phenological stages of the season. Control: untreated; Mid + Late: one application at pre-bunch closure; Early + Mid: two applications at flowering and one at pre-bunch closure; Early: two applications at flowering. Incidence was considered as the presence of *B. cinerea* sporulated mycelium. Presented values are means of six replicates. Mean values with the same letter are not significantly different ($P = 0.05$) according to Student's LSD *t*-test.

development in dry Mediterranean regions, where few vineyard epidemiological studies are available. Key phenological stages for BBR control have been identified, contributing to the development of disease management strategies in the region concerned, while new information is presented on potential secondary inoculum sources and their relationship with BBR at harvest.

Fungicide timing experiments conducted in two fields and over two seasons gave consistent information about the relevance of different phenological stages for *B. cinerea* epidemics. Most effective treatments consisted of field applications at flowering, with and without applications at pre-bunch closure or after veraison. These treatments, especially Early + Mid, were generally more effective than treatments applied from pre-bunch closure onwards, pointing to early season as essential for disease development compared with late season. Similar behaviour was observed in incidence and severity, although severity was less sensitive to treatment effect.

BBR disease pressure was lower in 2009 than in 2010, as evidenced by lower incidence and severity in the untreated controls during the first season. This difference may have been caused by meteorological conditions after veraison. In 2009, two iprodione applications at flowering (Early) were more effective in reducing incidence than mid- and late-season treatments. Nonetheless, when conditions were highly conducive in 2010, extra sprays at pre-bunch closure or late season were needed to observe significant differences with other effective treatments.

These results indicate that the most important stage for BBR development in the conditions tested is flowering, unless cooler temperatures and higher humidity in mid or late season lead to rapid development of the pathogen, making necessary later treatments. Other epidemiological studies also pointed to flowering as the most important stage for bunch rot development.[14,15,33–35] In contrast, other stages are reported

Table 2. Pearson correlations (*r*) between variables measuring secondary inoculum sources at veraison

	% Inc lat	Surface inoculum	% Inc ab flowers	% Inc ab fruits	% Inc calyptras
% Inc lat[a]	1	0.43	−0.07	0.40	−0.30
Surface inoculum[b]		1	0.04	0.02	−0.05
% Inc ab flowers[c]			1	0.49	−0.14
% Inc ab fruits[d]				1	−0.56
% Inc calyptras[e]					1

[a] Percentage incidence of *B. cinerea* latent infections on grape berries.
[b] Colony-forming units of *B. cinerea* per gram of berry samples.
[c] Percentage incidence of *B. cinerea* on aborted flowers trapped in the bunch.
[d] Percentage incidence of *B. cinerea* on aborted fruits trapped in the bunch.
[e] Percentage incidence of *B. cinerea* on necrotic calyptras trapped in the bunch.

Table 3. Summary of the multiple regression model for *Botrytis* bunch rot incidence (%) at harvest

Independent variables	VIF	Regression function	Model variables	*P*	R^2
% Inc lat[a]	1.87	% Incidence BBR = 33.74 + 2.80 (% Inc lat) + 0.88 (% Inc ab fruits) + 2.93 (% Inc calyptras)	% Inc lat	0.022	0.49
Surface inoculum[b]	1.37		% Inc calyptras	0.031	0.25
% Inc ab flowers[c]	1.66		% Inc ab fruits	0.001	0.20
% Inc ab fruits[d]	2.64				
% Inc calyptras[e]	1.53		Model	0.0002	0.95

[a] Percentage incidence of *B. cinerea* latent infections on grape berries.
[b] Colony-forming units of *B. cinerea* per gram of berry samples.
[c] Percentage incidence of *B. cinerea* on aborted flowers trapped in the bunch.
[d] Percentage incidence of *B. cinerea* on aborted flowers trapped in the bunch.
[e] Percentage incidence of *B. cinerea* on necrotic calyptras trapped in the bunch.

strongly to determine rot levels at harvest, depending on specific circumstances,[16,17,36–38] emphasising the importance of studying each particular region to define epidemiology and control strategies. The importance of flowering may also be increased in cultivars with compact bunch morphology, such as the Macabeu cultivar used in this study, which are considered to be more susceptible to BBR.[39–41]

The present results also provide information for the planning of spray applications. Two applications at flowering would be enough consistently to control the incidence and severity of BBR when late season remains dry, although the possibility of applying just one spray at 50% flowering in these conditions should also be explored. Moreover, if weather conditions in mid-season are conducive, the Early + Mid treatment would be the recommended one. However, as extra spray at pre-bunch closure or at late season improved control similarly, spraying can be delayed until conducive conditions for BBR development are confirmed later in the growing season, especially if the grape cultivar does not present compact bunch clusters. These findings are similar to those reported by Fermaud *et al.*[20] and contrary to the treatment practices of local growers, which frequently avoid early-season sprays, waiting until conditions become highly favourable for BBR (Late, Mid + Late and 7 days Preharvest treatments).

Quantification of the different secondary inoculum sources illustrated how *B. cinerea* develops in the vine prior to veraison and partly explained the efficacy of the different treatments controlling BBR. Flowering spray applications were able to reduce more than 87% of *B. cinerea* incidence in necrotic tissues or latent infections, showing high efficacy of those treatments controlling secondary inoculum and consequently bunch rot, as will be discussed later.

Latent infection levels detected in this work (4.4% incidence in untreated bunches) were similar to those detected in other studies on susceptible cultivars,[4,14,37] which presented average incidences of between 2 and 8%. Sanzani *et al.*[9] found very high incidence in three table grape cultivars (24–62%), while in another study conducted in Tunisian vineyards[21] the incidence of latent infections varied from 1.7 to 15.5%, depending on rainfall and RH registered during flowering. Latent infections may be produced during early flowering through conidial or mycelial infection of floral tissues, but also via fruit pedicel during early stages of berry development.[5] Nonetheless, there is little information available regarding the relative contribution of these two stages to the final percentage of latent infections detected at veraison. Keller *et al.*[14] found similar latent infection incidence at veraison in bunches inoculated at prebloom, full bloom and post-bloom, whereas Pezet *et al.*[4] also found higher incidence of latent infections at harvest in bunches inoculated at full bloom and, depending on the cultivar, in bunches inoculated post-bloom as well. However, in the present conditions, most of the latent infections originated during flowering, as latent infections were significantly reduced by early-season treatments and not by mid-season treatments.

Infections of necrotic tissues were mainly produced during flowering according to the complete 100% incidence reduction achieved by treatments consisting of flowering sprays (Early and Early + Mid). Only one spray application at pre-bunch closure also significantly reduced incidence, probably owing to suppression of *B. cinerea* in previously infected tissues.

Very low counts were registered when berry surface inoculum was evaluated, and no significant differences among samples were observed. This fact has also been reported in one of the few studies quantifying *B. cinerea* surface inoculum in vineyards,[42] indicating that more profuse sampling should be carried out in order to achieve more consistent data. However, the low density of conidia reported by other workers and other findings related to the infection process[11] suggest that BBR may not depend on surface inoculum density. The lack of correlation between bunch rot and surface inoculum counts in the present study supports this hypothesis.

The regression model based on untreated sample data considered three inoculum sources as explanatory variables of bunch rot incidence: % Inc lat, % Inc calyptras and % Inc ab fruits. These data confirm the significance of secondary inoculum and highlight the importance of *B. cinerea* incidence reductions in these sources. The incidence of latent infections is widely considered to determine the incidence of bunch rot,[4,9,13,21,43] although many early-season latent infections do not become active and its significance is not always clear.[5] According to the present results, necrotic calyptras in the bunch could play an important role, although no relation between necrotic calyptras and bunch rot was found by Chebil *et al.*,[21] while aborted fruits have been identified as an important inoculum source inside the bunch in previous works.[44,45]

To the present authors' knowledge there is no published research evaluating multiple secondary inoculum sources to correlate them with bunch rot. Some studies have identified aborted fruits as an important inoculum source,[45] whereas other authors have found significant correlations for other inoculum sources such as infected stamens,[9] carryover inoculum through seasons[13] or necrotic leaves and rachises in the canopy that eventually lead to latent infections and necrotic tissue infections.[45]

The present results showed a close relationship between three of the inoculum sources and incidence at harvest, evidenced by the high R^2 of the overall model, which indicates consistency of the results achieved. However, regression analysis was performed on a small sample number, and larger sampling would be necessary to deduce more consistent conclusions in further studies. In particular, for the regression function coefficients, values should be considered as a preliminary result rather than as an accurate predictive value.

It is also important to mention that the positive correlation between variables measuring inoculum at veraison and incidence of bunch rot at harvest was more evident owing to meteorological conditions registered from veraison to harvest in 2010. This suggests that the positive correlation between secondary inoculum and BBR may depend on favourable meteorological conditions for *B. cinerea* late in the season, which are more variable in Mediterranean-type climates. Some other studies have found that the relationship between secondary inoculum and BBR at harvest is inconsistent, depending on the season.[44–46]

Overall, the obtained epidemiological information clearly highlights key moments during the season in which *B. cinerea* suppression is essential for effective control of secondary inoculum and BBR at harvest. This information may also help to develop application recommendations for alternative strategies regarding synthetic fungicides to control BBR, such as natural products and biological control agents. Nonetheless, the efficacy of these other strategies depends on other factors that are often specific for each product;[47] for example, different biologically based treatments have recently been evaluated against *B. cinerea* incidence in necrotic tissues with different modes of action.[44] As infections in necrotic tissues in the present study were developed mainly during flowering (Figure 2), a proposed strategy to achieve reliable control would be the application of products protecting green floral tissues (chitosan, *C. sake*, Fungicover) during flowering, whereas *Ulocladium oudemansii* applications after flowering would be also desirable to suppress *B. cinerea* saprophytic colonisation developed between flowering and veraison. These treatments have shown high efficacy at harvest when combined with three applications post-veraison of the same products.[48]

The present study increases current epidemiological knowledge on BBR for dry Mediterranean and other similar regions. Flowering, in the conditions tested, represents the key phenological stage during the season for *B. cinerea* development as potential secondary inoculum and also to reduce bunch rot at harvest. Applications of fungicide during flowering were the most effective, whereas the addition of another application after veraison improved control when conditions were more favourable for BBR development. These results may help to reduce the number of applications compared with common practices based on regular timing or applications at four stages.

ACKNOWLEDGEMENTS

The authors are grateful to the University of Lleida for Carlos Calvo Garrido's PhD grant and research project on organic fruit production, to the European Union for their financial support (INTERREG REDBIO project) and to the Catalan government for Dr Elmer's travel assistance. They would also like to thank Dr Ricardo Blanco, of the statistics department of the Technical School of Agricultural Engineering (University of Lleida), for his valuable supervision of regression analysis.

REFERENCES

1 Ky I, Lorrain B, Jourdes M, Pasquier G, Fermaud M, Geny L *et al.*, Assessment of grey mould (*Botrytis cinerea*) impact on phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages. *Aust J Grape Wine R* **18**(2):215–226 (2012).
2 Holz G, Coertze S and Williamson B, The ecology of *Botrytis* on plant surfaces, in *Botrytis: Biology, Pathology and Control*, ed. by Elad Y, Williamson B, Tudzynski P and Delen N. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 9–27 (2004).
3 Nair NG and Allen RN, Infection of grape flowers and berries by *Botrytis cinerea* as a function of time and temperature. *Mycol Res* **97**:1012–1014 (1993).
4 Pezet R, Viret O, Perret C and Tabacchi R, Latency of *Botrytis cinerea* Pers.: Fr. and biochemical studies during growth and ripening of two grape berry cultivars, respectively susceptible and resistant to grey mould. *J Phytopathol* **151**(4):208–214 (2003).
5 Elmer PAG and Michailides TJ, Epidemiology of *Botrytis cinerea* in orchard and vine crops, in *Botrytis: Biology, Pathology and Control*, ed. by Elad Y, Williamson B, Tudzynski P and Delen N. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 243–272 (2004).
6 Leroch M, Kretschmer M and Hahn M, Fungicide resistance phenotypes of *Botrytis cinerea* isolates from commercial vineyards in south west Germany. *J Phytopathol* **159**(1):63–65 (2011).
7 Walker A-S, Micoud A, Remuson F, Grosman J, Gredt M and Leroux P, French vineyards provide information that opens ways for effective resistance management of *Botrytis cinerea* (grey mould). *Pest Manag Sci* **69**(6):667–678 (2013).
8 Crane M, Norton A, Leaman J, Chalak A, Bailey A, Yoxon M *et al.*, Acceptability of pesticide impacts on the environment: what do United Kingdom stakeholders and the public value? *Pest Manag Sci* **62**(1):5–19 (2006).
9 Sanzani SM, Schena L, De Cicco V and Ippolito A, Early detection of *Botrytis cinerea* latent infections as a tool to improve postharvest quality of table grapes. *Postharvest Biol Technol* **68**:64–71 (2012).
10 Jaspers MV, Seyb AM, Trought MCT and Balasubramaniam R, Overwintering grapevine debris as an important source of *Botrytis cinerea* inoculum. *Plant Pathol* **62**(1):130–138 (2013).
11 Holz G, Gütschow M, Coertze S and Calitz FJ, Occurrence of *Botrytis cinerea* and subsequent disease expression at different positions on leaves and bunches of grape. *Plant Dis* **87**(4):351–358 (2003).
12 Shtienberg D, The contribution of epidemiological research to plant disease management. *Austral Plant Pathol* **36**(6):510–515 (2007).
13 Nair NG, Guilbaud-Oulton S, Barchia I and Emmett R, Significance of carry over inoculum, flower infection and latency on the incidence of *Botrytis cinerea* in berries of grapevines at harvest in New South Wales. *Aust J Exp Agric* **35**(8):1177–1180 (1995).
14 Keller M, Viret O and Cole FM, *Botrytis cinerea* infection in grape flowers: defense reaction, latency, and disease expression. *Phytopathology* **93**(3):316–322 (2003).
15 Petit AN, Vaillant-Gaveau N, Walker AS, Leroux P, Baillieul F, Panon ML *et al.*, Determinants of fenhexamid effectiveness against grey mould on grapevine: respective role of spray timing, fungicide resistance and plant defences. *Crop Prot* **29**(10):1162–1167 (2010).
16 Viret O, Bloesch B, Dubuis PH and Gindro K, Epidemiology and control strategies against grey mould (*Botrytis cinerea*). *Rev Suisse Vitic Arboric Hortic* **42**(3):162–167 (2010).
17 de Kock P and Holz G, Application of fungicides against postharvest *Botrytis cinerea* bunch rot of table grapes in the Western Cape. *S Afr J Enol Vitic* **15**(2):33–40 (1994).
18 Agnew RH, Mundy DC and Balasubramaniam R, Effects of spraying strategies based on monitored disease risk on grape disease control and fungicide usage in Marlborough, in *57th Annual Meeting of the New Zealand Plant Protection Society*, ed. by Zydenbos SM. New Zealand Plant Protection, Hamilton, New Zealand, pp. 30–36 (2004).
19 Raynal M, Debord C, Guittard S, Vergnes M, Griaud K, Fernandez N *et al.*, Ne traiter que si necessaire: le point sur les travaux de modelisation des risques phytosanitaires appliques par l'IFV au domaine de la protection du vignoble. *Prog Agric Vitic* **126**(24):571–581 (2009).
20 Fermaud M, Roudet J, Sauris P, Druelle L, Blanc G and Lagouarde P, Implementation of a low-input fungicide strategy in relation to the pattern of grey mold development. Abstract Book of the XV International *Botrytis* Symposium, Cadiz, Spain (2010).
21 Chebil S, Roudet J, Dubos B and Ghorbel A, Table grapes in Tunisia. Optimization of protection with regard to grey mould. *Arboric Fruit* **574**:i–v (2003).

22 Cortesi P, Zerbetto F and Vercesi A, Biology and epidemiology of *Botrytis cinerea* Pers. on grape. *Inform Fitopatol* **41**(6):45–48 (1991).
23 D'Arcangelo MEM, Storchi P, Egger E, Orlandini S, Mancini M, Marta AD, Phenological phases, microclimate and modelling of grapevine grey mould at farm scale. *Italus Hortus* **14**(3):247–252 (2007).
24 Gil E, *Tratamientos en Viña: Equipos y Técnicas de Aplicación*. Edicions UPC, Barcelona, Spain (2003).
25 Servei de Sanitat Vegetal, Plagues: la podridura grisa (*Botrytis cinerea*), in *Catalunya Rural i Agrària*. DAAM, Barcelona, Spain, pp. 39–40 (2002).
26 Diez-Navajas AM and Ortiz-Barredo A, Downy, powdery mildew and *Botrytis* risk assessment by climate parameters, pest and spores monitoring in Rioja Alavesa vineyard (Northwest of Spain). *IOBC/WPRS Bull* **67**:21–29 (2011).
27 Gil E, Llorens J, Landers A, Llop J and Giralt L, Field validation of DOSAVIÑA, a decision support system to determine the optimal volume rate for pesticide application in vineyards. *Eur Food Res Technol* **35**(1):33–46 (2011).
28 *Agricultural Crop Productions*. [Online]. FAOSTAT. Available: http://faostat.fao.org/site/636/DesktopDefault.aspx?PageID=636#ancor [14 May 2013].
29 *Anuario de Estadística. Superficies y Producciones de Cultivos*. [Online]. MARM. Available: http://www.magrama.gob.es/estadistica/pags/anuario/2011/AE_2011_13_11_01_01.pdf [14 May 2013].
30 *Climatologia. L'Urgell 1971–2000*. [Online]. Servei Meteorologic de Catalunya. Available: http://www20.gencat.cat/docs/meteocat/Continguts/Climatologia/climatologies_comarcals/documents/Urgell.pdf [4 February 2013].
31 *R.E.D. Facts. Iprodione. Prevention, Pesticides and Toxic Substances*. [Online]. USEPA, EPA-738-F-98-017. Available: http://www.epa.gov/oppsrrd1/REDs/factsheets/2335fact.pdf [14 May 2013].
32 Edwards SG and Seddon B, Selective media for the specific isolation and enumeration of *Botrytis cinerea* conidia. *Lett Appl Microb* **32**(2):63–66 (2001).
33 Viret O and Keller M, Influence of flower infection of grapevine by *Botrytis cinerea* Pers. on latency and bunch rot at harvest. Abstracts of the XII *Botrytis* Symposium, Reims, France, p. 47 (2000).
34 Michailides TJ, Morgan PA, Felts D and Peacock W, Infection of California table grapes and detection and significance of symptomless latent infection by *Botrytis cinerea*. *Abstracts of the XII* Botrytis *symposium*, Reims, France (2000).
35 van Rooi C and Holz G, Fungicide efficacy against *Botrytis cinerea* at different positions on grape shoots. *S Afr J Enol Vitic* **24**(1):11–15 (2003).
36 Pearson RC and Riegel DG, Control of *Botrytis* bunch rot of ripening grapes – timing applications of the dicarboximide fungicides. *Am J Enol Vitic* **34**(3):167–172 (1983).
37 Latorre BA, Lillo C and Rioja ME, Effect of timing on the efficacy of fungicide treatments applied against *Botrytis cinerea* of grapevine. *Cien Inv Agr* **28**(2):61–66 (2001).
38 Zitter S and Wilcox W, Effect of spray timing and fungicide physical mode of action on the development of *Botrytis* bunch rot (BBR) on grapes. *Phytopathology* **95**(6):S118–S119 (2005).
39 Hed B, Ngugi HK and Travis JW, Relationship between cluster compactness and bunch rot in Vignoles grapes. *Plant Dis* **93**(11):1195–1201 (2009).
40 Alonso-Villaverde V, Boso S, Santiago JL, Gago P and Martinez MC, Relationship between susceptibility to *Botrytis* bunch rot and grape cluster morphology in the *Vitis vinifera* L. cultivar Albarino. *Int J Fruit Sci* **8**(4):251–265 (2008).
41 Fermaud M, Liminana JM, Froidefond G and Pieri P, Grape cluster microclimate and architecture affect severity of *Botrytis* rot of ripening berries. *IOBC/WPRS Bull* **24**(7):7–9 (2001).
42 Duncan RA, Stapleton JJ and Leavitt GM, Population dynamics of epiphytic mycoflora and occurrence of bunch rot of wine grapes as influenced by leaf removal. *Plant Pathol* **44**(6):956–965 (1995).
43 McClellan WD and Hewitt WB, Early *Botrytis* rot of grapes: time of infection and latency of *Botrytis cinerea* Pers. in *Vitis vinifera* L. *Phytopathology* **63**(9):1151–1157 (1973).
44 Calvo-Garrido C, Viñas I, Elmer PA, Usall J and Teixidó N, Suppression of *Botrytis cinerea* on necrotic grapevine tissues by early-season applications of natural products and biological control agents. *Pest Manag Sci* DOI: 10.1002/ps.3587.
45 Seyb A, *Botrytis cinerea* inoculum sources in the vineyard system. *PhD Thesis*, Lincoln University, Christchurch, New Zealand (2004).
46 Wolf TK, Baudoin A and Martinez-Ochoa N, Effect of floral debris removal from fruit clusters on *Botrytis* bunch rot of Chardonnay grapes. *Vitis* **36**(1):27–33 (1997).
47 Elad Y, Mechanisms involved in the biological control of *Botrytis cinerea* incited diseases. *Eur J Plant Pathol* **102**:719–732 (1996).
48 Calvo-Garrido C, Elmer PAG, Viñas I, Usall J, Bartra E and Teixidó N, Biological control of *Botrytis* bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1. *Plant Pathol* **62**:510–519 (2013).

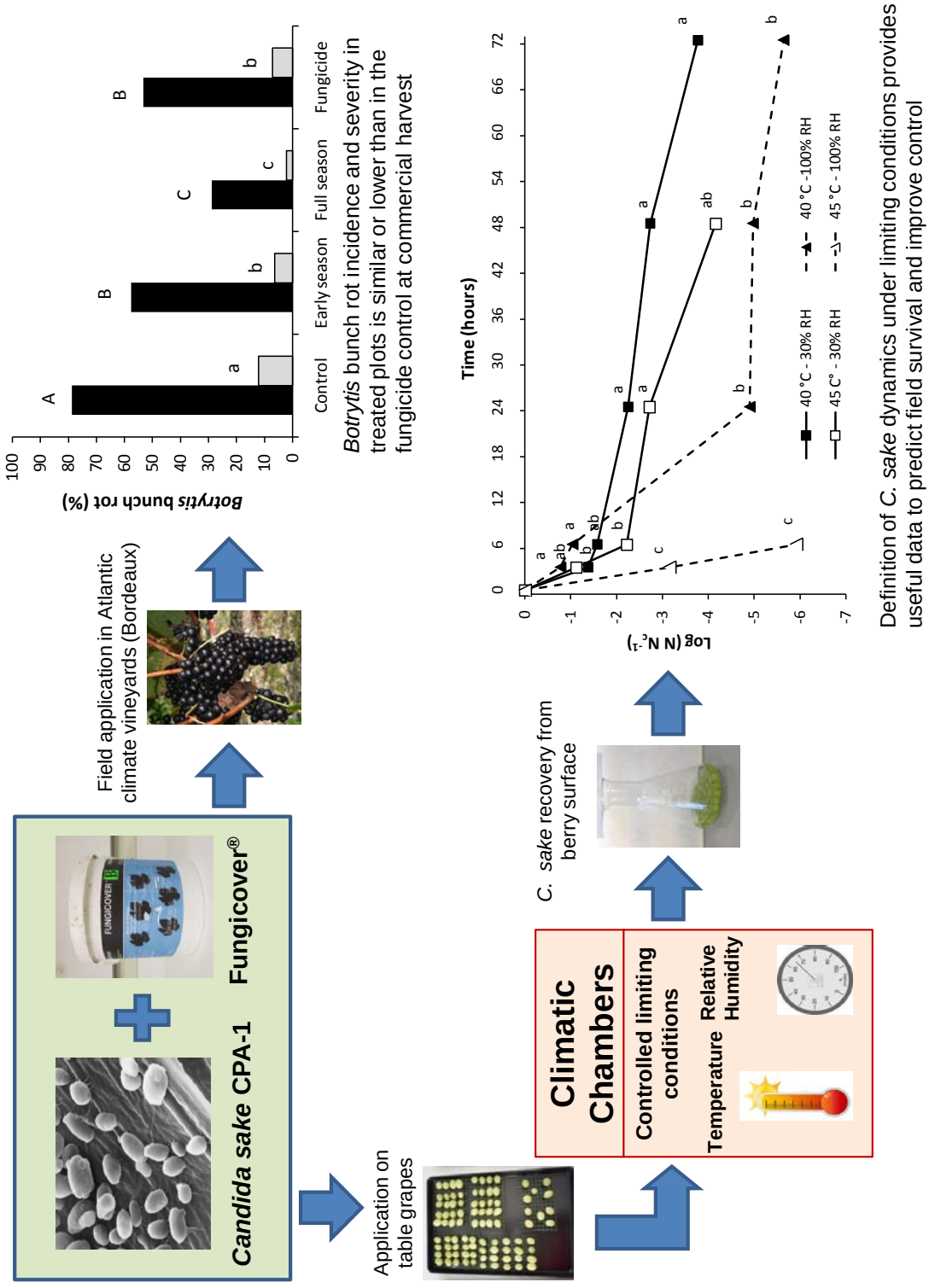
CAPÍTULO 6

Biological control of *Botrytis bunch* rot with *Candida sake* CPA-1 in Atlantic climate vineyards and the effect of controlled limiting conditions of temperature and humidity on its survival

C. Calvo-Garrido, N. Teixidó, J. Roudet, I. Viñas, J. Usall and M. Fermaud

Enviado a: ***Biological Control***

Graphical Abstract



Biological control of *Botrytis* bunch rot with *Candida sake* CPA-1 in Atlantic climate vineyards and the effect of controlled limiting conditions of temperature and humidity on its survival

Authors

C. Calvo-Garrido [a]; N. Teixidó, [b]; J. Roudet [c]; I. Viñas [a]; J. Usall [b]; M. Fermaud [c]

[a] *Food Technology Department, Lleida University, XaRTA-Postharvest, Agrotecnio Center, Rovira Roure 191, 25198 - Lleida, Catalonia, Spain*

[b] *IRTA, XaRTA-Postharvest, 191 Rovira Roure, 25198-Lleida, Catalonia, Spain*

[c] *UMR 1065 Santé & AgroEcologie du Vignoble (SAVE) - ISVV - Centre INRA de Bordeaux -71 av. Edouard Bourlaux - BP 81, 33883 - Villenave d'Ornon cédèx - France*

Abstract

The application of biological control agents (BCA) is a promising method to control *Botrytis* bunch rot of grapes (BBR). Particularly, *Candida sake* CPA-1 has previously shown to effectively control BBR. Efficacy of yeast antagonists is dependent on BCA survival. However, few studies have evaluated the effect of abiotic factors affecting BCA survival, such as Temperature (T) or Relative humidity (RH). In this study, efficacy of *C. sake* ($5x10^7$ CFU mL^{-1}), applied with the additive Fungicover (FC; 50 g L^{-1}) was tested against BBR in laboratory and field trials in the Atlantic climate region of Bordeaux. The study also evaluated survival of *C. sake* under simulated T and RH regimes. Two or five applications of *C. sake* plus FC along the growing season significantly reduced BBR severity at harvest (48% and 82% compared to control, respectively). Similar reductions were achieved on table grapes inoculated with selected virulent strains (75% compared to control). Survival pattern of *C. sake* was described at 40 ^{0}C and 45 ^{0}C combined with 30% and 100% of RH, identifying a remarkable decrease during the first 24 h. Moreover, the occurrence of a establishment period, prior to limiting conditions, increased survival ($P < 0.05$) on berry surface. Populations maintained higher levels in the simulated Atlantic conditions, supporting the field population dynamics results. The results confirm efficacy of *C. sake* plus FC under favourable climatic conditions for BBR development, while survival studies show useful data to improve biological control based on yeast BCA applications, such as *C. sake* CPA-1.

Keywords

abiotic factors, extreme conditions, fatty acids, fungal disease, grapevine, *Vitis vinifera*

1. Introduction

Biological control by antagonist microorganisms has been extensively studied in the last decades and is regarded as a promising alternative to the use of synthetic fungicides to control fruit pathogens (Droby et al., 2009; Nicot, 2011). Among these pathogens, *Botrytis cinerea* is the causing agent of the *Botrytis* bunch rot of grapes, which is an important disease in vineyards responsible for economic losses in wine and table grapes. In the Bordeaux winegrowing region, for example, BBR may deplete quantitatively the entire yield if meteorological conditions are very conducive to epidemic development and, qualitatively, it has been shown recently a significant loss of wine sensory quality, perceptible from a disease threshold as low as 5% of rotted berries at harvest (Ky et al., 2012). The epidemiological development following *B. cinerea* infection is complex, notably for BBR, because it is dependent on various factors which have been associated with multiple infection pathways (Elmer and Michailides, 2004). Among the key factors, there are: i) the genetic structure of the *B. cinerea* population (Martinez et al., 2008, 2005); ii) the grape berry ontogenic resistance associated with the fruit developmental stage (Deytieux-Belleau et al., 2009); iii) the grapevine susceptibility related to plant vegetative vigour (Valdés-Gómez et al., 2008) and important interactions between the pathogen and insect vectors such as *Lobesia botrana*, the European Grape Berry Moth "EGBM" (Fermaud and Lemenn, 1992) and *Thrips obscuratus* (Fermaud and Gaunt, 1995).

Promising studies report effective preharvest disease control by commercialised biological control agent (BCA) products and other microorganisms in developmental stages (Elmer and Reglinski, 2006; Nally et al., 2012; Parry et al., 2011). Furthermore, postharvest applications of several BCAs have also demonstrated to be effective during storage of table grapes (Romanazzi 2011). The yeast antagonist *Candida sake* CPA-1, applied with the additive Fungicover® (FC) has shown to effectively reduce BBR incidence and severity at harvest in field experiments in dry Mediterranean conditions (Calvo-Garrido et al., 2013; Cañamás et al., 2011), whereas previous research also reported efficacy against postharvest diseases of pome fruit through pre- and post-harvest applications (Teixidó et al., 1999, 1998b). Spatial and nutrient competition on fruit surfaces is considered to be the main mechanism by which *C. sake* CPA-1 brings about disease suppression, as described for other antagonist yeast and yeast-like fungi (Droby et al., 2009; Filonow, 1998; Jijakli, 2011; Lima et al., 1997). This mode of action requires the presence of BCA cells at high concentration on fruit tissues along the time and a minimal concentration of 10^4 UFC cm^{-2} is needed to maintain consistency of control, making survival of BCA populations to be a crucial factor (Andrews, 1992).

BCA survival is affected by abiotic factors such as temperature (T), relative humidity (RH), UV radiation (Lahlali et al., 2011; Magan, 2001; Teixido et al., 2010). The influence of these factors can be reduced during controlled postharvest storage. However, under field conditions, populations are subjected to meteorological fluctuations, daily temperature and RH changes as well as periods of limiting conditions interfering with BCA survival. Especially in hot and dry summer climates, such as the Mediterranean region type, days with maximum T over 35 ^{0}C are frequent and RH can drop below 30% during the day, corresponding to very challenging situations for the survival of a yeast BCA like *C. sake*, which has an optimal growth temperature of 25 ^{0}C in culture medium (Teixidó et al., 1998c).

In addition to the survival constrains that can interfere drastically with BCA efficacy, meteorological conditions can also affect the pathogen-BCA interaction in the field. For instance, *B. cinerea* germination, *in vitro* growth and infection of grape berries is optimal at 20 °C but very reduced at temperatures over 25 °C (Martinez et al., 2003, 2005). Consequently, differences in T and RH during the growing season are known as key factors determining BBR epidemic development and final rot levels at harvest (Elmer and Michailides, 2004). On the other hand, BCA performance is modified by environmental conditions, as suggested some of the few studies investigating RH and T effect on pathogen and BCA relationship (Agra et al., 2012).

Concerning the population monitoring of antagonist yeast and yeast-like fungi, population dynamics have been assessed during storage, following postharvest applications on a variety of fruit commodities: mainly in pome fruit (Jijakli, 2011; Lima et al., 2003; Manso and Nunes, 2011; Viñas et al., 1998), but also in sweet cherries (Ippolito et al., 2005; Tian et al., 2004) and citrus (Teixidó et al., 2001). Other studies have also evaluated field survival of these BCAs to control diseases during the season (Guetsky et al., 2002; Lima et al., 2002) or following pre-harvest applications to control fruit pathogens during storage (Benbow and Sugar, 1999; Ippolito and Nigro, 2000; Lahlali et al., 2009; Teixidó et al., 1998b). In grapes, BCA population studies include the evaluation of field survival after postharvest applications of *Aureobasidium pullulans* and *Candida oleophila* (Lima et al., 1997), *Candida guillermondii* and *Acremonium cephalosporium* (Zahavi et al., 2000), *Metschnikowia fructicola* (Karabulut et al., 2003), *Metschnikowia pulcherrima* and *Pichia guilliermondii* (Kinay and Yildiz, 2008), *Cryptococus laurentii* (Meng and Tian, 2009), the developing yeast-like fungi BCA-L1 (Parry et al., 2011) and *C. sake*, evaluated in conventional and organic managed vineyards (Calvo-Garrido et al., 2013; Cañamás et al., 2011).

In contrast, there are few publications focused on the direct effect of key abiotic factors such as T and RH on BCAs survival. *In vitro* response of *C. sake* to water, temperature and pH stress has been previously studied (Teixidó et al. 1998c), whereas Lahlali et al. (2008) established a model for the survival of *Pichia anomala* and *C. oleophila* strains, exposing populations on treated apples to different temperatures (5, 15 and 25 °C) and RH (75% and 98%). Furthermore, *C. oleophila* was also tested in extreme conditions of water activity and RH (Lahlali and Jijakli, 2009). However, there are no similar studies on grapes and none has evaluated *In vivo* survival of a BCA under simulated climatic regimes or under limiting T and RH regimes in controlled conditions. This information could be valuable to predict survival and hence improve efficacy of treatments, especially, in hot and dry regions, which are expected to increase according to most of climate change scenarios.

In the present paper, the efficacy of *C. sake* CPA-1 plus Fungicover against *B. cinerea* was tested in laboratory and in a Bordeaux vineyard, under typical Atlantic meteorological conditions, where field populations of the BCA were monitored. In addition, an exhaustive study on *C. sake* CPA-1 survival was carried out in order to evaluate yeast population dynamics on grape berries following different regimes of simulated climatic conditions and limiting conditions of T and RH.

2. Materials and Methods

2.1 Yeast and fungal material

Three pathogenic *B. cinerea* strains (213, 344 and 351) were selected from the collection of INRA (UMR 1065 SAVE), Bordeaux. The strains have been characterised as II-*vacuma* for the strain 351) and II-*transposa* for both the 213 and 344 strains. They were selected according to their marked aggressiveness ranging from high virulence (213) to intermediate-high virulence (351 and 344) compared to other *B. cinerea* strains from the same collection (Martinez et al., 2003). Stock cultures were maintained on solid malt agar (MA) medium (15 g L^{-1} Cristomalt, Materne, France and 20 g L^{-1} of agar) and then subcultured on MA at 21 °C (± 1°C) in the dark.

The strain CPA-1 of *C. sake* is deposited in the Colección Española de Cultivos Tipo (CECT-10817) at the University of Valencia, Burjassot, Spain. *C. sake* was used for experiments as a formulated product developed in the IRTA research centre LOCATED IN Lleida (Catalonia, Spain), following cell production and formulation methods described by Cañamás et al., (2011), and then stored at 5 °C (± 1°C) in INRA facilities prior to application of treatments..

For all field and laboratory experiments, *C. sake* was applied together with the additive Fungicover (Biodúrcal S.L., Granada, Spain). Its composition is a combination of different fatty acids (mainly lauric, palmitic and stearic acids) in an aqueous-alcoholic solution and is used as coating agent for *C. sake* field applications since it has demonstrated to improve survival of the BCA (Cañamás et al., 2011) and also to reduce BBR itself (Calvo-Garrido et al., 2013).

2.2 Efficacy of C. sake *and Fungicover treatments against* B. cinerea *infection in controlled laboratory conditions*

The combination of *C. sake* CPA-1 plus the additive FC was tested in its capacity to control berry infections following *B. cinerea* artificial inoculations using mycelial plugs. Infection severity was compared to an untreated control and a treatment with Fungicover itself.

Table grapes (cv. Italia) were washed for 15 minutes in continuous tap-water flow to remove particles and synthetic fungicide residues. Then, single apparently sound berries were cut from bunches with scissors, keeping the pedicel attached (average berry maturity = 13.1 °Brix, measured by refractometry).

The experimental design included three replicates per treatment with one replicate unit consisting of 15 berries placed on a grid. The treatments were i) untreated berries (Control), ii) berries sprayed with *C. sake* at 5 x 10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1} (CS+FC) and iii) berries sprayed with Fungicover at 50 g L^{-1} alone (FC). To favour *C. sake* establishment prior to inoculation with *B. cinerea*, after fruit drying, each replicate unit was introduced in a high humidity chamber (HHC) consisting of a plastic box (22 x 13 x 4 cm) containing a sterile absorbent paper in the base with 50 ml of sterile deionised water and incubated 18 hours at 21°C (± 1°C) and 100% RH.

Mycelial plugs (4 mm diameter) were cut from the edge of 7-day-old colonies of *B. cinerea* and placed on the berries with the mycelium facing the fruit surface using a sterile scalpel. Once inoculation was carried out and boxes closed again to create HHC, the 27 replicate units (three replicates by three treatments by three *B. cinerea* isolates) were introduced in an incubator (EX-111; TABAI ESPEC CORP, Osaka, Japan) at constant temperature of 21 °C (± 0.5°C) and 100% RH in the dark until symptom assessments. After 5, 7, 11 and 14 days of incubation, rot severity was visually scored in each berry as the percentage of berry surface showing the typical brown colour following *B. cinerea* infection. Average rot severity of each replicate unit was calculated as the mean severity of the 15 berries in each HHC. Based on the average rot severity in each replicate unit plotted over the time, the area under disease progress curve was calculated for subsequent statistical analysis.

2.3 Efficacy of field applications of C. sake *and Fungicover in Bordeaux vineyards*

2.3.1 Experimental field site

In 2012, a field efficacy assay was conducted in an INRA experimental vineyard (*Vitis vinifera* L.) located near Bordeaux ("Grande Ferrade", Villenave d'Ornon). The vineyard of Merlot cultivar was planted in 1991 on typical gravely soil and was grafted onto '101-14' rootstock. The planting density was approximately 5350 vines ha^{-1} with a row by vine spacing of 1.70 m x 1.10 m and a north-south row orientation. The experimental vineyard was not treated with any specific anti-*Botrytis* fungicide.

2.3.2 Anti-*Botrytis* treatments and experimental design

The growing season was divided in five key phenological stages from flowering to harvest: 50% flowering (04 Jun. 2012), 50% flowering + 15 days (18 Jun. 2012), Pre-bunch closure (06 Jul. 2012), Veraison (13 Aug. 2012) and 21 days before harvest (06 Sep. 2012). Applications of *C. sake* plus the additive Fungicover were carried out at those stages during the whole season or focused, only, on the early part of the growing season ("Full season" and "Early season" treatments). Efficacy of the treatments was compared to an untreated control and to a usual synthetic fungicide program in the Bordeaux region. Details of concentrations, active ingredients applied and timetable of spray applications are summarised in Table 1.

Table 1 Field treatments with *Candida sake* CPA-1 and Fungicover applied to control *Botrytis* bunch rot in Merlot wine grapes

Treatment[a]	50% flowering	50% flowering + 15 days	Pre bunch closure	Veraison	21 days before harvest
Control	-	-	-	-	-
Full season	CS+FC	CS+FC	CS+FC	CS+FC	CS+FC
Early season	CS+FC	-	CS+FC	-	-
Fungicide	Fenhexamid	-	Cyprodinil + Fludioxonil	-	-

[a] **CS+FC**: *C. sake* CPA-1 at 5 x 10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1}; **Fenhexamid**: Fenhexamid 750 g ha^{-1}; **Cyprodinil + Fludioxonil**: 1200 g ha^{-1} of Cyprodinil (37.5%) plus Fludioxonil (25%)

Each replicate unit in the field experiment consisted of seven adjacent vines, where the first and the last vines were buffer vines, second and third plants were used for *C. sake* population sampling, and the remaining three vines were used to assess BBR at the end of the season. Replicate units were sorted in a completely randomized block design with five replicates per treatment.

C. sake was applied as the formulated product previously described in the laboratory experiment. Field applications were carried out with an electric backpack sprayer (F200 model, INFACO s.a.s., Cahuzac sur Vère, France) by applying treatments until runoff on the inflorescences or grape bunches only.

2.3.3 Bunch rot assessment

At the end of the season, two assessments of BBR were carried out: 1) at commercial harvest time (26 Sept. 2012) and 2) one week later, when grape bunches were over-ripen (04 Oct. 2012). Incidence and severity of bunch rot were both assessed on 50 bunches per replicate unit, scored individually. Incidence was measured as the percentage of bunches with visual and typical *B. cinerea* rot symptoms. Bunch rot severity was measured visually as the percentage of *B. cinerea*-rotted berries per bunch.

2.3.4 Evaluation of *C. sake* populations on floral and fruit organs

C. sake populations on vine tissues were monitored along the growing season in treated plots. Random sampling of grapevine tissues was carried out using sterile clippers on the basis of four replicates corresponding to the first 4 blocks of the original five-block experimental design, collecting samples from the second and third vines of each replicate unit. In the Full season treated plots, flowers or berries were sampled a) once the tissue surface was dry just after one

treatment application and b) one hour before the next treatment application. In the Early season treatment, populations on berries were assessed at the end of the season only. Sampling was repeated twice (27 Sep. and 4 Oct.) to gain consistency and precision of the final average value, resulting from eight replicates.

At flowering, BCA populations were recovered from 2 g of floral organs that were collected from 8 bunches per replicate unit. Samples where then immersed in 20 mL of phosphate buffer. At pre-bunch closure, 40 pea-sized berries from 20 bunches per unit plot were weighed and then immersed in 50 mL of phosphate buffer. In samplings after veraison, 20 berries from 10 bunches were also weighed and then immersed in 50 mL of phosphate buffer.

Once the sampled tissues had been in 250-mL Erlenmeyer flasks with phosphate buffer, they were shaken for 20 min at 150 r.p.m on a rotary shaker and then sonicated for 10 min in an ultrasonic bath (Branson® 2510, Branson Ultrasonics Corp., Danbury, Connecticut, USA). After serial dilutions of the washing solution, 100 µL aliquots were plated onto NYDA plates (NYDA: nutrient broth, 8 g L^{-1}; yeast extract, 5 g L^{-1} ; dextrose, 10 g L^{-1}; and agar, 15 g L^{-1}) supplemented with streptomycin sulphate (0.5 g L^{-1}). Duplicate plates were incubated in the dark at 25 °C and colonies were visually recognised and counted, based on their morphological characteristics, after 48 hours. Data was collected as CFU mL^{-1} and expressed as CFU g^{-1} of tissue sample.

2.3.5 **Quantification of *Lobesia botrana* larvae in grape bunches**

At the end of the season, five bunches were selected randomly from each replicate unit plot, regardless of the *Botrytis* rot level. Samples were collected from all replicate unit plots of the field experiment, excluding fungicide treated ones. Then, each batch of five bunches was immersed and agitated in three litres of brine (NaCl, ca 170 g L^{-1} of water) for 20 to 30 min in a bucket, as previously described by Fermaud (1998). The number of *L. botrana* larvae of the third generation per five bunches was counted at the brine surface and then expressed as "Number of *L. botrana* larvae per 100 bunches". The sampling and counting process was carried out twice (04 Oct. 2012 and 09 Oct. 2012) for higher consistency of results.

2.3.6 Meteorological data

A weather station (CIMEL 516i, CIMEL Electronique, Paris, France), belonging to the INRA meteorological service network, was placed next to the experimental vineyard recording data of main meteorological variables in hourly intervals. Mean daily values were calculated of Mean Temperature (T), Maximal Temperature (T_{max}), Mean Relative Humidity (RH) and Accumulative Rainfall (Rf) were calculated afterwards.

2.4 Studies on C. sake *CPA-1 survival in climatic chambers*

2.4.1 General methodology

Five experiments were conducted under controlled conditions to evaluate different features of *C. sake* survival on the grape berry surface according to different temperature and RH regimes. These regimes were generated using two climatic chambers (EX-111 model, TABAI ESPEC CORP, Osaka, Japan and PGR14 model, CONVIRON Ltd, Winnipeg, Canada) that were calibrated to adjust temperature and RH values in the two chambers at the same level.

The common methodology for all experiments was as follows. Mature table-grape berries were used after they were washed for 15 minutes in a continuous tap-water flow to remove particles and synthetic fungicide residues. Ten single apparently sound berries were cut keeping the pedicel attached and placed onto a grid constituting a replicate unit. 1) The first step on the experimental procedure was to apply *C. sake* plus FC treatments with a hand sprayer, applying to three replicate units per treatment. Fruit were let dry and then samples were introduced in HHC's as those described in section 2.2. 2) As a second step, the treated berries were incubated in HHC's at 21°C (±1°C) and 100% RH in dark to favour establishment of *C. sake* cells on the berry surface. 3) The third step corresponded to exposure to controlled T and RH regimes in dark, with different temperature x RH x duration combinations. 4) Then, at the indicated moments (depending on the experiment), recovery and quantification of *C. sake* culturable cells on the 10 berries of each replicate unit was carried out, proceeding as above-described (section 2.3.4) for the field efficacy experiment.

Specific conditions of the five studies in climatic chambers were designed as modifications of the first three steps of the general methodology, to study the effect of those stages in *C. sake* survival. Three replicate units per treatment consisting of ten berries each were used in all the survival experiments.

2.4.2 Effect of constant limiting T and RH conditions on *C. sake* survival

In order to evaluate the reduction of *C. sake* populations on grape berries in limiting conditions, treated berries were exposed to constant regimes combining high temperature with low or high RH conditions.

Thompson seedless table grapes were sprayed with *C. sake* CPA-1 at 5 x 10^7 CFU L^{-1} together with Fungicover at 50 g L^{-1} and then incubated in HHC's at 21°C (± 1°C) during 12-18 hours. Four treatments corresponding to four different regimes of limiting conditions in a 72 hours period were then tested: 1) 40 °C and 30% RH, 2) 40 °C and 100% RH, 3) 45 °C and 30% RH and 4) 45 °C and 100% RH. During the same 72 hours period, another set of samples was incubated at 21°C (± 1°C) and 100% RH as a Control treatment. Evaluation of *C. sake* populations on berries was performed after 0, 3, 6, 24, 48 and 72 hours. Results of the *C. sake* s reduction were finally expressed, for each sample time, as Log (N N_c^{-1}), where N = populations in treated sample (UFC g^{-1}) and N_c = Mean value of the population in the three replicates of the Control treatment (UFC g^{-1}). The experiment was repeated twice at 40 °C and 30% RH to increase

consistency of data on this treatment, which was considered as representative standard of hot and dry limiting field conditions.

2.4.3 Effect of the additive Fungicover on *C. sake* survival under constant limiting conditions

The fatty-acid based natural product FC had shown to improve *C. sake* CPA-1 survival in field conditions (Cañamás et al., 2011), but the mechanism by which this additive protects the yeast remains unclear. Thus, in this study, *C. sake* was applied alone or associated with different concentrations of FC to evaluate if *C. sake* survival under constant limiting conditions of temperature and RH was affected by the FC dose.

Three treatments were evaluated based on application of *C. sake* at 5 x 10^7 CFU L^{-1} on table grapes (cv. Sugraone): *C. sake* alone (CS), *C. sake* plus Fungicover at 25 g L^{-1} (CS+FC25) and *C. sake* plus Fungicover at 50 g L^{-1} (CS+FC50). Once treated, samples were incubated in HHC's at 21°C (± 1°C) during 12-18 hours and then exposed to a 72 hours period at 40 °C and 30% RH. During the same period, as Control treatments, a similar set of samples was incubated at 22 °C (± 1°C) and 100% RH. Evaluation of *C. sake* populations on berries was performed after 0, 6, 24, 48 and 72 hours. Results of the *C. sake* population counts were finally expressed for each sample time as Log (CFU g^{-1}).

2.4.4 Effect of establishment period prior to limiting conditions on *C. sake* survival

The effect on *C. sake* survival capacity of different incubation periods under optimal conditions (establishment period) before a period of constant limiting conditions was tested. For that purpose, grape berries (cv. Sugraone) were treated with *C. sake* CPA-1 at 5 x 10^7 CFU L^{-1} plus Fungicover at 50 g L^{-1} and then incubated at 21 °C (± 1°C) and 100% RH during 0, 24 or 48 hours (0h, 24h and 48h treatments). Just after the incubation period, samples were exposed to a 48 hours period of limiting conditions at 40 °C and 30% RH. Evaluation of *C. sake* populations on berries was performed 0 and 48 hours after the beginning of the limiting conditions period. Results of the *C. sake* populations before limiting conditions period were expressed as Log (CFU g^{-1}). Population reductions after the limiting conditions exposure were finally expressed as Log ($N\ N_0^{-1}$) where N = CFU g^{-1} of *C. sake* after exposure to limiting conditions period and N_0 = average value of CFU g^{-1} of *C. sake* before the limiting conditions period.

2.4.5 *C. sake* survival under Atlantic and Mediterranean simulated climatic regimes

To evaluate the survival of *C. sake* populations on grape berries under Atlantic or dry Mediterranean climatic regimes, two simulated night/day regimes were designed using meteorological data from representative weather stations from each climatic region (Merignac Airport in Bordeaux region; IRTA weather station placed in an experimental vineyard in Lleida region). Average values of T and RH variables during the central part of the growing season were calculated using meteorological data from July 1st to August 31st (2006 to 2011) in the two weather stations.

Simulated day regimes (BDX and LDA treatments for Bordeaux and Lleida simulated conditions, respectively) consisted of a combination of the Average Maximal daily temperature and the Average Minimal daily RH in each region. Simulated night regimes consisted of a

Table 2 Temperature and RH conditions of the Atlantic and Mediterranean simulated climatic regimes applied to *C. sake* populations in survival studies

	Day regime (15 hours)	Night regime (9 hours)
Atlantic climate Bordeaux, France (BDX)	27 °C – 43% RH	16 °C – 93% RH
Dry Mediterranean climate Lleida, Catalonia, Spain (LDA)	31 °C – 39% RH	16.5 °C – 82% RH

combination of the Average Minimal daily temperature and the Average Maximal daily RH in each region. Temperature and RH values of the simulated night/day regimes are described in Table 2. Day and night duration was 15 and 9 hours respectively, according to approximate duration of day and night in both regions on August 1st.

Sugraone table grapes were treated with *C. sake* CPA-1 at 5 x 10^7 CFU L^{-1} plus Fungicover at 50 g L^{-1} and incubated in HHC's at 21 °C (± 1°C) during 12-18 hours. Then samples were introduced in the climatic chambers programmed with the simulated climatic regimes (BDX and LDA), while another set of samples was maintained in HHC at 21 °C (± 1°C) and 100% RH during the whole experiment being considered as Control treatment. *C. sake* populations were recovered after 0, 1, 4, 7 and 15 days after the start of the simulated conditions.

2.4.6 Effect of the adaptation to simulated climatic regimes on *C. sake* survival to constant limiting conditions

The objective was to test whether *C. sake* populations that have been exposed to different climatic conditions survive differently to a subsequent period of constant limiting conditions. For that purpose, after the application of *C. sake* CPA-1 at 5 x 10^7 CFU L^{-1} plus Fungicover at 50 g L^{-1} on table grapes (cv. Sugraone) and the incubation in HHC's at 21 °C (± 1°C) during 12-18 hours, samples were introduced in climatic chambers programmed with the simulated climatic regimes (BDX and LDA treatments, Table 2) during five days. Then, samples coming from both Atlantic and dry Mediterranean simulated conditions were put together under limiting conditions (40 °C and 30% RH) for 72 hours. Populations of *C. sake* were measured at 0, 6, 24, 48 and 72 hours after the start of the limiting conditions exposure. Population reductions were finally expressed for each sample time as Log ($N N_0^{-1}$) as previously described.

2.5 Statistical analysis

Data were analysed by ANOVA and significant treatment differences ($P < 0.05$) were determined using Tukey test. LSD Student's *t*-test was used in the analysis of efficacy experiment comparing treatments to a untreated control. To improve homogeneity of variances, *L. botrana* larvae counts were transformed [Square root (x+1)] and CFU data of *C. sake* CPA-1 population counts were log-transformed prior to ANOVA. Data analysis was performed using JMP8 software (SAS Institute Inc., NC, U.S.A.).

3. Results

3.1 Efficacy of C. sake *and Fungicover treatments against* B. cinerea *infection in controlled laboratory conditions*

Symptom development curve of the berry infection produced by *B. cinerea* mycelial plugs in the three tested treatments is presented in Figure 1. Data includes mean severity results of the three *B. cinerea* strains tested (351, 344, 213), since interactions between treatment effect and strains were not significant and hence data could be analysed together. However, there were significant differences ($P < 0.05$) in mean severity produced by the 213 strain (II-*transposa*) compared to the 344 and 351 strains (II-*transposa* and II-*vacuma* respectively). The area under disease progress curve produced by the 213 strain was 33% higher or more than the area of the 344 or the 351 strains, indicating elevated aggressiveness of 213 compared to the other two (data not shown).

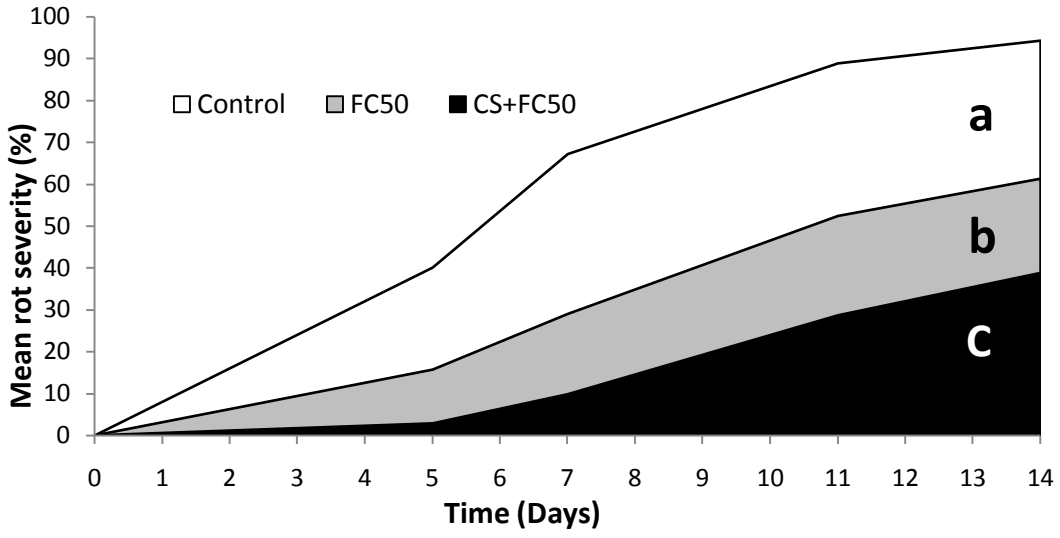


Figure 1 Mean severity of rot in table grapes treated with *C. sake* and Fungicover before challenge inoculation with *Botrytis cinerea* mycelial plugs (351, 213 and 344 strains). Grape berries (cv. Italia) were: not treated (Control), treated with *C. sake* CPA-1 at 5 x 10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1} (CS+FC) or treated with Fungicover alone at 50 g L^{-1} (FC). After incubation at 21-23 ºC at 100% RH, berries were inoculated and then incubated at 21 °C and 100% RH during 14 days. All values are means of three replicate units per treatment * strain. Mean values of the calculated area under disease progress curve connected by the same letter are not significantly different ($P < 0.05$) according to LSD Student's *t* –test.

Mean severity in untreated berries increased gradually arriving to 94% at 14 days after inoculation. Severity in treated berries was significantly lower ($P < 0.05$) and differences between both treatments were significant too ($P < 0.05$). The CS+FC and FC treatments reduced the area under the curve 75% and 47% respectively (Figure 1), compared to the untreated control. Nonetheless, maximal severity reduction by CS+FC and FC treatments was achieved at five (92% and 60% reduction respectively) and seven (85% and 56% respectively) days after inoculation.

3.2 Efficacy of field applications of C. sake *and Fungicover in Bordeaux vineyards*

3.2.1 Control of BBR at harvest

Figure 2 summarises results of incidence and severity of *Botrytis* bunch rot in the field experiment conducted on an experimental Merlot vineyard. In the rot assessment at commercial harvest (Figure 2a) incidence and severity were high in untreated plots, reaching 78% and 12%, respectively. All treatments significantly reduced ($P < 0.05$) incidence and severity of BBR. Two applications of *C. sake* and Fungicover at key phenological stages (Early season treatment) reduced incidence by 26% and severity by 48% compared to the untreated control, and reductions were not significantly different from those achieved by the Fungicide treatment (32% and 41% of incidence and severity, respectively). The Full season treatment was the most effective treatment, controlling bunch rot incidence by 63% and severity by 82%.

One week after commercial harvest maturity (Figure 2b), *Botrytis* bunch rot incidence and severity in untreated plots increased up to 99% and 39% respectively. Only the Full season and Fungicide treatments significantly reduced ($P < 0.05$) incidence by 13% and 18%, respectively. However, all treatments were able to reduce BBR severity by 34% (Early season), 77% (Full season) or 74% (Fungicide), although Early season treatment showed significantly lower ($P < 0.05$) efficacy than the Full season and Fungicide treatments.

3.2.2 Population dynamics of *C. sake* during field efficacy experiment

Populations of the yeast antagonist *C. sake* were very high on flowers, recovering more than 7 Log (CFU g^{-1}) after the spray application at 50% flowering (Figure 3). Then, *C. sake* decreased to 5.7 Log (CFU g^{-1}) prior to next application. At flowering plus 15 days, populations were reloaded up to 6.7 Log (CFU g^{-1}) and decrease rate was low until pre bunch closure application.

When *C. sake* plus Fungicover mixture was applied on berries, from pre bunch closure onwards, populations always stayed over 5 Log (CFU g^{-1}) after the spray applications and did not significantly decrease ($P < 0.05$) between applications. The decrease rate between these samples was very low and the sprays neither increased significantly ($P < 0.05$) *C. sake* numbers on berry surface compared to the populations present just before the application. The *C. sake* population recovered at harvest from Early season plots was 4.9 Log (CFU g^{-1}), which was not different ($P < 0.05$) from those on Full season berries, even if last spray application was carried out at pre bunch closure, two months and three weeks before harvest.

3.2.3 Quantification of *Lobesia botrana* larvae in grape bunches

The results of the *L. botrana* counts showed elevated incidence in untreated Merlot bunches at harvest, reaching 123 larvae / 100 bunches (Figure 4). However, incidence of *L. botrana* was 72% lower (34 larvae / 100 grape bunches; $P < 0.05$) in the Full season treatment plots compared to control, which suggests that treatments with *C. sake* and Fungicover, applied to control BBR, are also reducing grape berry moth development. In contrast, the Early season treatment presented similar incidence than the untreated control.

3.2.4 Meteorological data

Meteorological conditions before veraison (01/06/2012 to 13/08/2012) were characterised by mean T = 20.2 °C, mean T_{max} = 25.9 °C, mean RH = 65.9% and accumulative Rf = 120.0 mm. During the late season period, after veraison (14/08/2012 to 10/10/2012), mean T was 20.0 °C, mean T_{max} = 26.3 °C, mean RH was 66.3% and accumulative Rf was 73.5 mm.

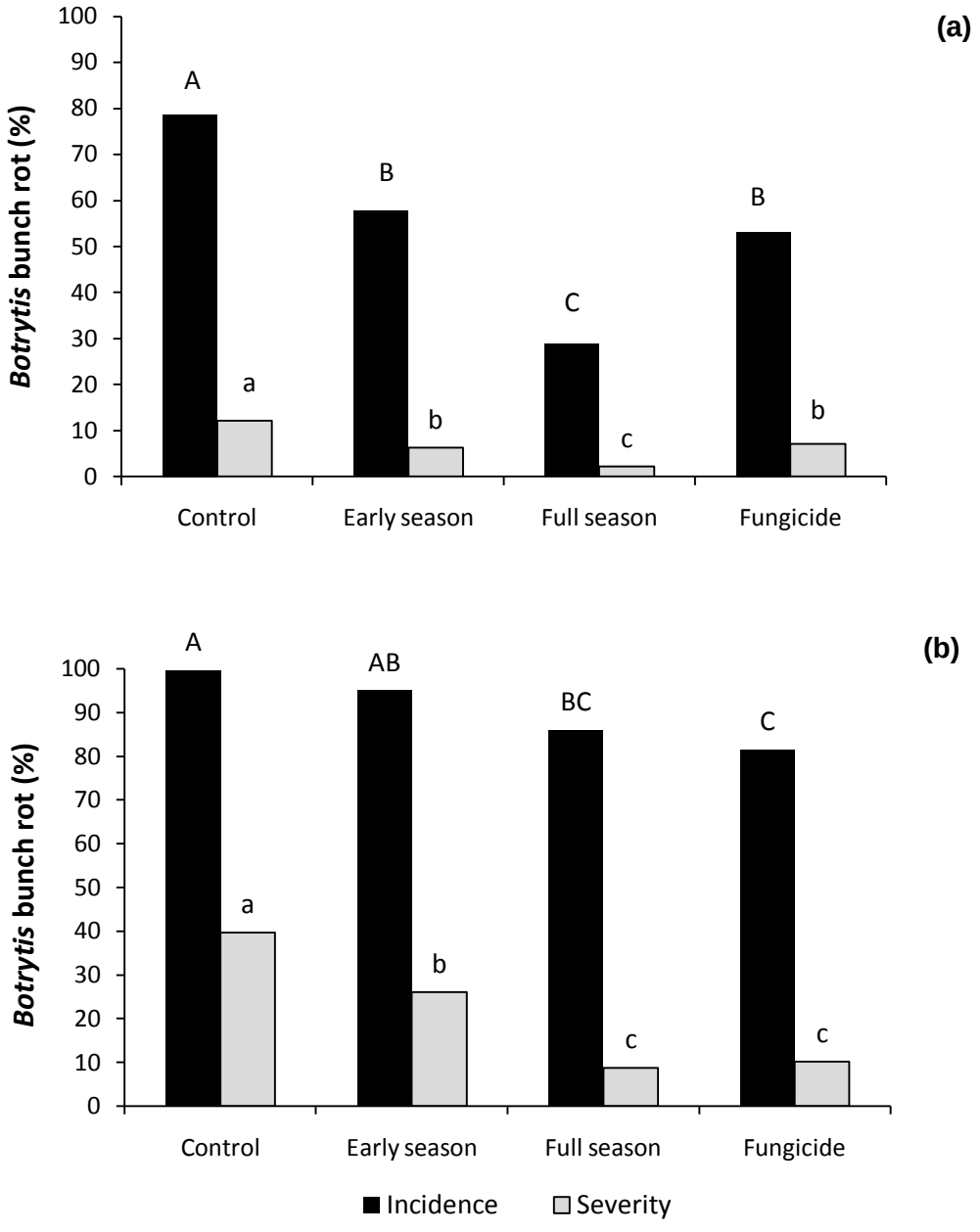


Figure 2 Incidence and severity of *Botrytis* bunch rot at harvest in an experimental vineyard cv. Merlot in Bordeaux. Bunch rot was assessed over 50 bunches per replicate plot at a) commercial harvest date (26/09/2012) and b) one week after commercial harvest (04/10/2012). *C. sake* CPA-1 at 5 x 10^7 CFU/g plus Fungicover at 50 g/L was applied two times (Early season) or five times (Full season) at key phenological stages. Control: untreated; Fungicide: One application of Fenhexamid at 50% flowering (Dose: 750 g ha^{-1}) and one application of Cyprodinil (75%) + Fludioxonil (25%) at pre bunch closure (Dose: 1200 g ha^{-1}). All values are means of five replicate units. Mean values of incidence or severity linked by the same letter are not significantly different ($P < 0.05$) according to LSD Student's *t* -test

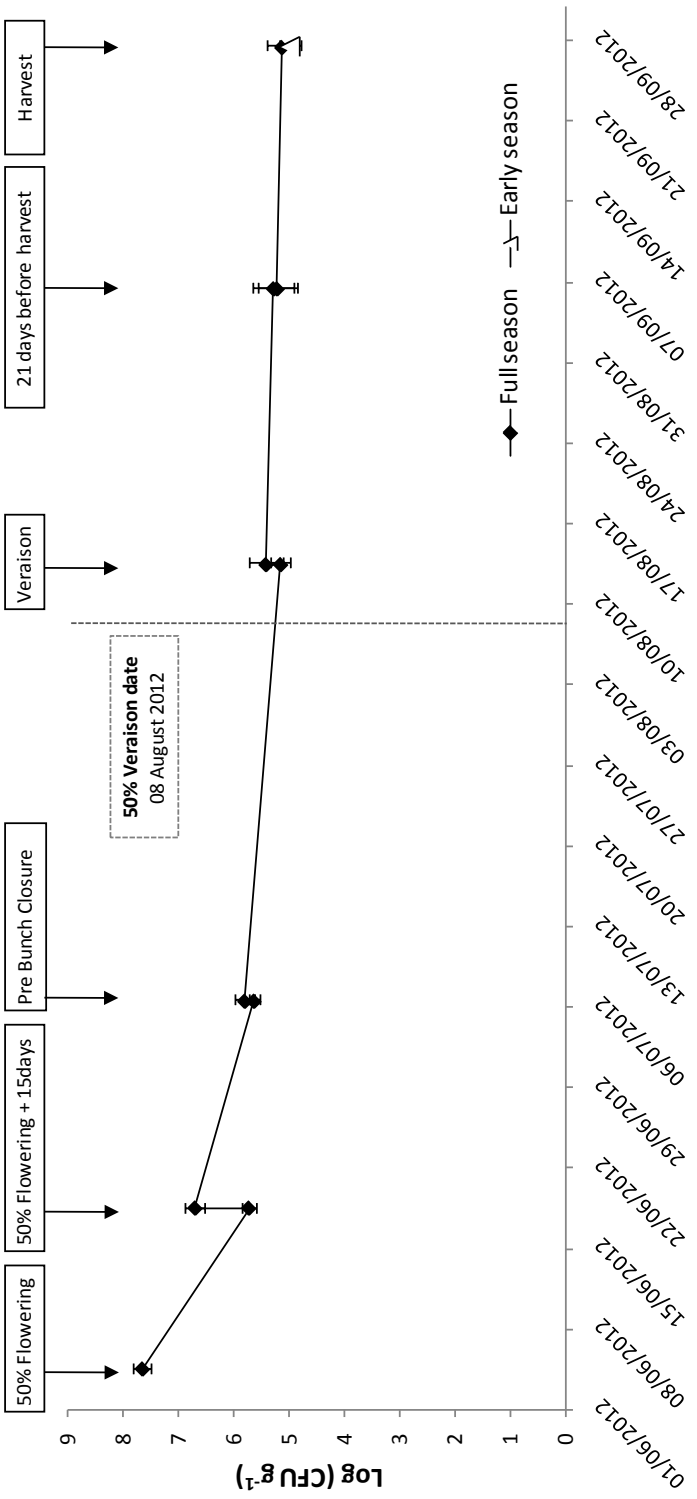


Figure 3 Population dynamics of *Candida sake* CPA-1 on grape vine tissues from an experimental vineyard cv. Merlot in Bordeaux. *C. sake* CPA-1 at 5 x 10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1} was applied five times at key phenological stages along the season (Full season), or two times (Early season) at 50% flowering and pre bunch closure. Flower or berry samples were taken after spraying and again just prior to the next spray application. Populations in Early season treatment were only assessed at harvest and indicated value is mean of eight replicates. CFU values are per gram of tissue sampled and were log-transformed. Values are means of four replicates and error bars represent sample Standard Deviation.

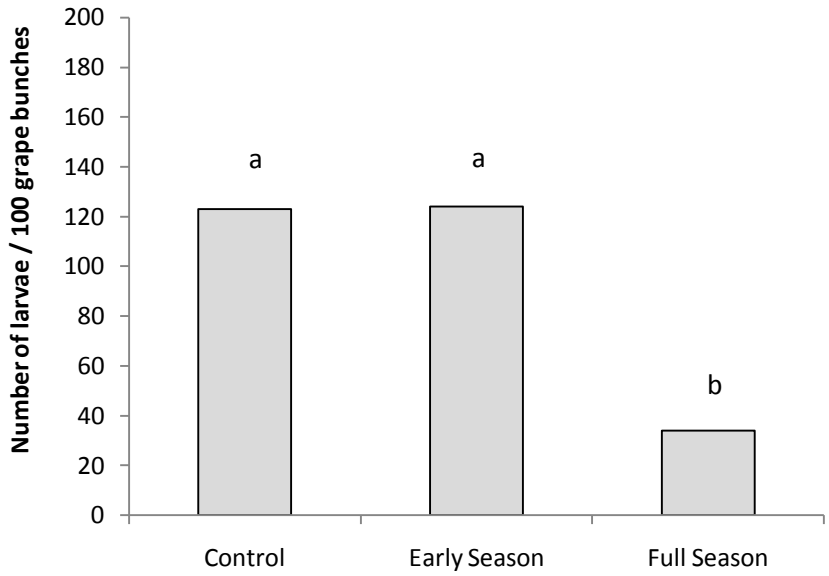


Figure 4 Incidence of *Lobesia botrana* larvae in Merlot grape bunches from an experimental vineyard in Bordeaux at harvest. *C. sake* CPA-1 at 5 x 10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1} was applied two times (Early season) or five times (Full season) at key phenological stages. Control: untreated. Five bunches per replicate unit were collected and immersed in a saturated NaCl solution to recover *L. botrana* larvae present in mature bunches. All values are means of ten replicate units. Larvae counts data were transformed [Square root (x+1)] prior to ANOVA. Mean values linked by the same letter are not significantly different ($P < 0.05$) according to LSD Student's *t* -test.

3.3 Studies on C. sake *CPA-1 survival in climatic chambers*

3.3.1 Effect of constant limiting T and RH conditions on *C. sake* survival

The *C. sake* populations on treated grape berries decreased under the four T and RH regimes tested (Figure 5). When limiting temperatures were combined with low humidity conditions (40 °C – 30%; 45°C – 30%), populations' decline pattern was similar in both treatments and no significant differences ($P < 0.05$) were detected at any sampling time between them. Nonetheless, results showed a trend in which *C. sake* recovered populations were generally lower in samples maintained at 45 °C. After 48 hours exposure, population decreased 2.7 Log units (40 °C – 30%) or 4.1 Log units (45°C – 30%), while after 72 hours at 40 °C and 30% decreased 3.7 Log units. Another interesting trend in samples at 30% RH indicated that decrease rate was large and important during the first six hours of exposure and then populations stabilised and continued decreasing in a lower rate during the rest of the exposure period.

In samples exposed to limiting temperature and high RH (40 °C – 100%; 45°C – 100%), population decline was higher than reductions observed in the low RH regimes. At 40 °C and 100% RH, populations decreased 5.6 Log units after 72 hours while, since the first 24 hours of exposure, reduction was significantly higher ($P < 0.05$) compared to reduction in the 40 °C – 30% treatment. However, populations decrease during the first six hours at 100% RH was similar or significantly lower (sample at 3 hours exposure; $P < 0.05$) than at 40 °C and 30% RH.

At 45 °C and 100% RH, decrease was linear and very intense during the first hours of the exposure period, losing 5.9 Log units after six hours. No *C. sake* populations could be recovered on the 24 hours samples. Population reductions were always significantly different ($P < 0.05$) between the 45°C – 100% and the 45°C – 30% treatments.

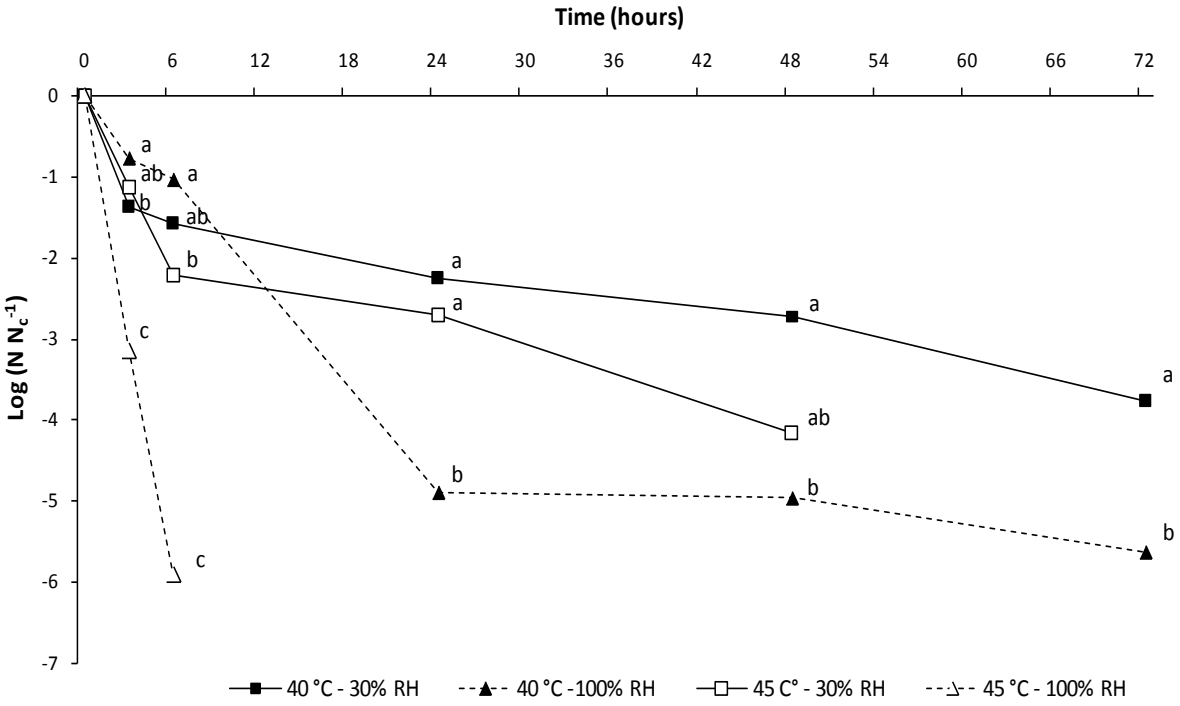


Figure 5 Reduction of *Candida sake* CPA-1 populations on grape berries exposed to limiting conditions of Temperature and RH. Thompson seedless berries were treated with *C. sake* CPA-1 at 5 x 10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1}, then incubated at 21-23 °C and 100% RH before being exposed to regimes of T and RH. N = Populations in treatment sample (CFU g^{-1}) and N_c = Mean value of the populations (CFU g^{-1}) on berries incubated at 21-23 °C and 100% RH during the same period. Represented values are means of three replicates except 40 °C – 30% samples, which are means of six replicates. Mean values linked by the same letter are not significantly different ($P < 0.05$) according to Tukey test.

3.3.2 Effect of Fungicover on *C. sake* survival to constant limiting conditions

Population dynamics of *C. sake* applied with different concentrations of Fungicover and subjected to optimal and limiting conditions of T and RH are shown in Figure 6. Initial populations at the start of exposure ranged from 5.4 to 5.7 Log (CFU g^{-1}) and no significant differences were detected at that point.

Populations slightly increased in samples incubated in HHC at 21 °C during 72 hours (CS, CS+FC25 and CS+FC50; black symbols) and the final *C. sake* concentration on berry surface ranged from 5.9 to 6.1 Log (CFU g^{-1}). No significant differences were detected between these three treatments at any sampling time.

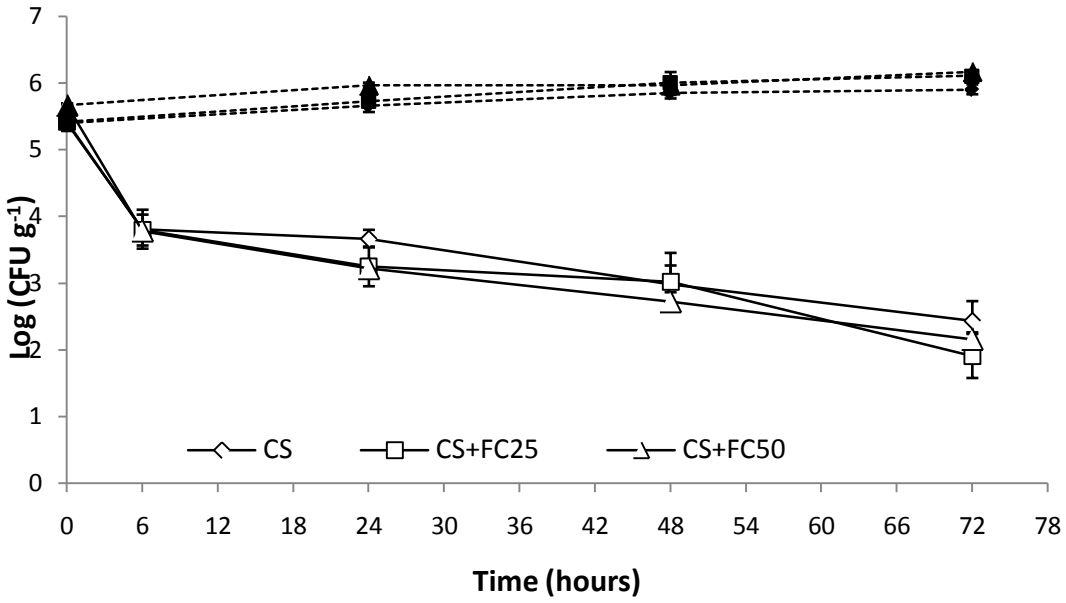


Figure 6 *Candida sake* population dynamics on grape berries treated with combinations of the yeast and different concentrations of Fungicover. Values represent recovered *C. sake* populations from grape berry surface along a 72 hours period in optimal conditions (21-23 °C and 100% RH; Dotted lines and black symbols) or limiting conditions (40 °C and 30 % RH; solid lines and white symbols). Berries (cv. Sugraone) were treated with *C. sake* CPA-1 at 5 x 10^7 CFU mL^{-1} alone (CS), combined with Fungicover at 25 g L^{-1} (CS+FC25), or combined with Fungicover at 50 g L^{-1} (CS+FC50). Values are means of three replicates and error bars represent sample Standard Deviation.

In all the samples exposed to limiting conditions (CS, CS+FC25 and CS+FC50; white symbols) population decreased in a similar pattern for all the Fungicover concentrations tested. Decrease pattern was also similar to those observed in Figure 5, with a remarkable population reduction during the first six hours and a second stage with a lower reduction rate. Final *C. sake* population ranged from 1.9 to 2.4 Log (CFU g^{-1}), which means a reduction of approximately 4 Log units compared to populations in the samples incubated under optimal conditions. No significant differences were observed between treatments at any sample time, indicating no effect of the Fungicover dose on *C. sake* survival under the constant conditions evaluated.

3.3.3 Effect of the establishment time prior to constant limiting conditions on C. sake *survival*

Populations of *C. sake* significantly increased on berry surface when they were incubated at 21 °C and 100% RH for 24 or 48 hours (Figure 7a) after BCA application. In the first 24 hours, populations significantly augmented ($P < 0.05$) from 4.7 to 5.7 Log (CFU g^{-1}). Between 24 hours and 48 hours of incubation, *C. sake* populations also increased ($P < 0.05$) from 5.7 to 5.9 Log (CFU g^{-1}).

The decrease in *C. sake* populations on berries after 48 hours exposure to limiting conditions (40°C – 30% RH), when berries had been previously incubated in optimal conditions (21 °C – 100% RH) for 0, 24 and 48 hours is shown in Figure 7b. The populations decreased 3.4 and 3.0 Log units in samples previously incubated 0 and 24 hours respectively. This reduction was significantly higher ($P < 0.05$) than the reduction observed in samples incubated for 48 hours prior exposure, which decreased 2.1 Log units.

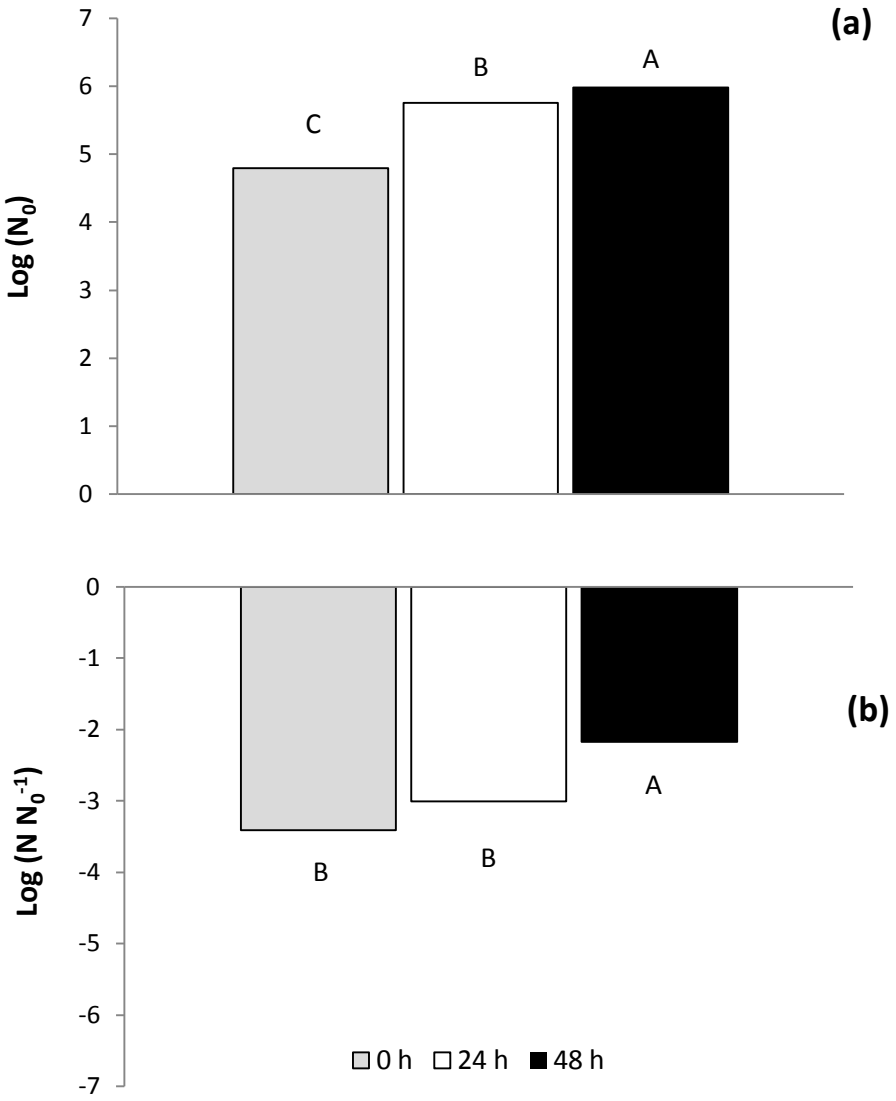


Figure 7 Effect of establishment time prior to exposure to limiting conditions of T and RH on *Candida sake* survival. a) Populations on berry surface after incubation at 21-23 °C and 100% RH during 0, 24 or 48 hours (0h, 24h, 48h); b) Reduction of populations on berry surface at the end of a 48 hours period of limiting conditions (40 °C – 30%), when populations had been previously incubated at 21-23 °C and 100% RH during 0, 24 or 48 hours (0h, 24h, 48h). Treatment mixture applied on grape berries (cv. Sugraone) consisted of *C. sake* CPA-1 at 5 x 10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1}. N = CFU g^{-1} of *C. sake* after exposure to limiting conditions period, N_0 = average value of CFU g^{-1} of *C. sake* before the start of limiting conditions period. Values are means of three replicates. Mean values connected by the same letter are not significantly different ($P < 0.05$) according to Tukey test.

3.3.4 *C. sake* survival under Atlantic and Mediterranean simulated climatic regimes

The *C. sake* populations behaved differently under the regimes of T and RH tested, as evidenced in Figure 8. At constant 21 °C and 100% RH (Control), yeast population on berries at the beginning was 6.2 Log (CFU g^{-1}) and remained stable between 6.1 and 6.2 Log (CFU g^{-1}) during the entire assay. When samples were exposed to the simulated climatic regimes, a significant decrease in both treatments (BDX and LDA) was observed in the first 24 hours. Then, populations continued decreasing until 4 days of exposure to 5.4 and 4.9 Log (CFU g^{-1}) under simulated Atlantic (BDX) and Mediterranean (LDA) simulated climatic regimes, respectively. The *C. sake* concentration on berries at that sample time was significantly different

($P < 0.05$) among the three treatments. After 4 days, the populations in all conditions tested remained stable until the end of the assay (15 days after the start of exposure), and populations ranged between 5.4 - 5.5 (BDX), or 4.9 - 4.8 (LDA), maintaining around 0.5 Log unit difference among treatments.

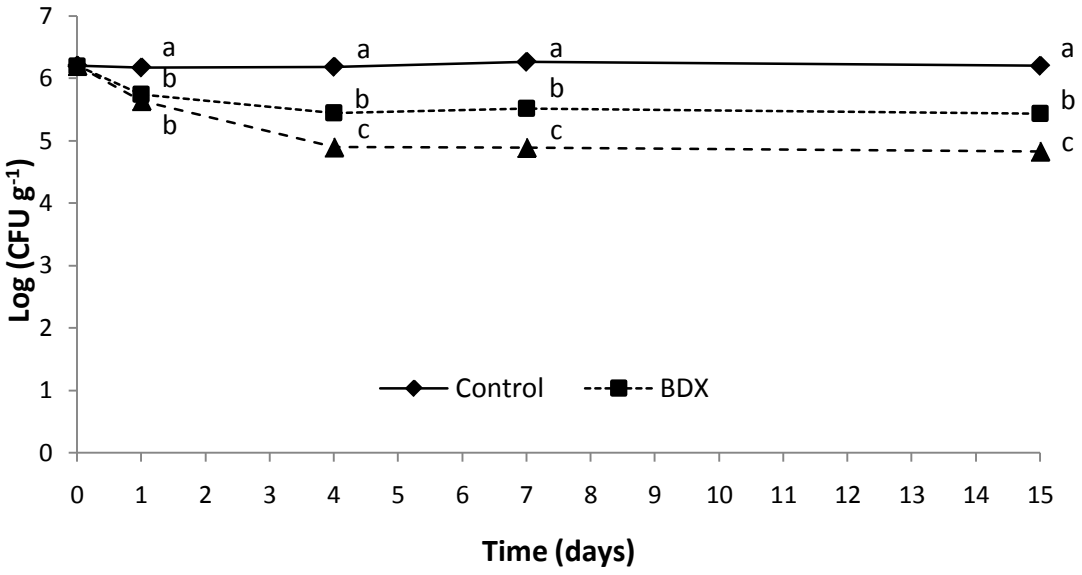


Figure 8 Population dynamics of *Candida sake* CPA-1 on grape berries exposed to simulated Atlantic and Mediterranean climatic regimes. Sugraone grape berries were treated with *C. sake* CPA-1 at 5 x 10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1}, incubated at 21-23 °C and 100% RH and then exposed during 15 days to: constant 21-23 °C and 100% RH conditions (Control), simulated Atlantic conditions (BDX) or simulated Mediterranean conditions (LDA). Values are means of three replicates. Mean values connected by the same letter are not significantly different ($P < 0.05$) according to Tukey test.

3.3.5 Effect of the adaptation to simulated climatic regimes on *C. sake* survival to constant limiting conditions

After 72 hours exposure to limiting conditions, populations decreased between 1.1 and 1.6 Log units, in samples previously exposed to Mediterranean (LDA) and Atlantic (BDX) simulated regimes, respectively (Figure 9). Decrease in samples not exposed to simulated regimes was 3.7 Log units (Control).

Differences in decrease between LDA and BDX treatments were not significant ($P < 0.05$) at any sample time. However, population decrease in LDA samples trended to be less remarkable during the first six hours of exposure and final average population Log (CFU g^{-1}) was 0.5 Log units higher than in BDX samples.

The *C. sake* populations previously adapted to both climatic regimes survived significantly better ($P < 0.05$) than those previously incubated in optimal conditions.

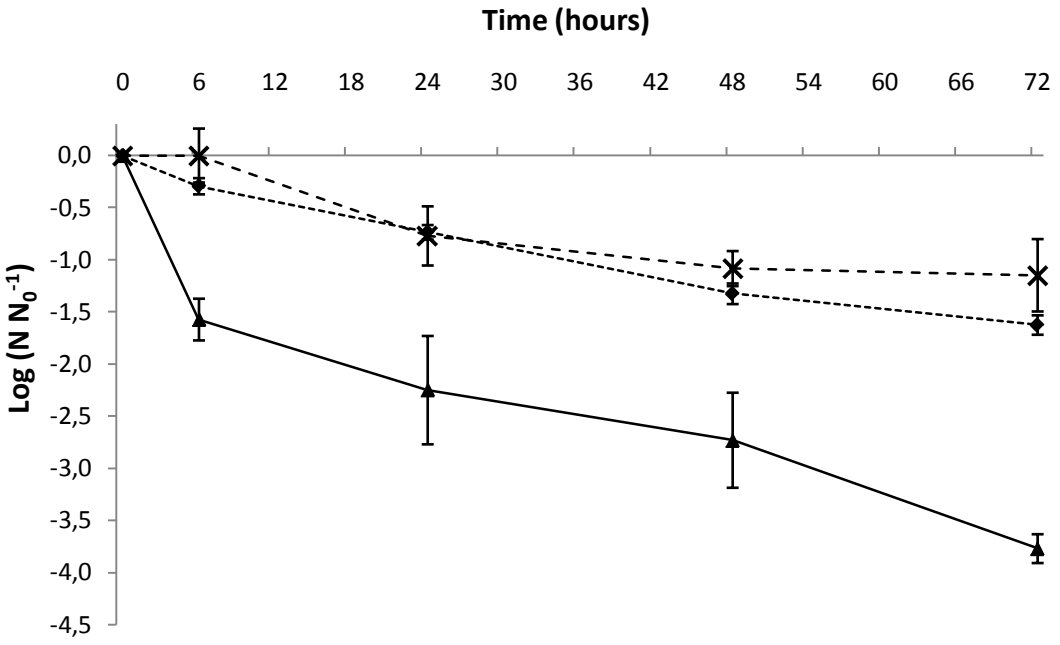


Figure 9 Reduction of *Candida sake* CPA-1 populations exposed to limiting conditions on grape berries previously exposed to simulated climatic regimes. Berries (cv. Sugraone) were treated with *C. sake* CPA-1 at 5 x 10^7 CFU mL^{-1} plus Fungicover at 50 g L^{-1}, then incubated at 21-23 °C and 100% RH. Prior to limiting conditions period, samples were exposed during five days to simulated Atlantic (BDX) or Mediterranean (LDA) climatic regimes. Control samples were not exposed to any simulated regime. Limiting conditions period consisted of 72 hours at 40 °C – 30 % RH. N = CFU g^{-1} of *C. sake* after exposure to limiting conditions period, N_0 = average value of CFU g^{-1} of *C. sake* before the start of limiting conditions period. Values are means of three replicates and error bars represent sample Standard Deviation.

4. Discussion

An important factor contributing to the success of a BCA in field applications is the ability of the BCA to adapt to different climatic conditions and show efficacy against the target pathogen in a wide range of conditions. For the first time, in this study, *C. sake* CPA-1 treatments were evaluated under Atlantic conditions, conducive to BBR, in order to complement and confirm previous studies in Catalan vineyards with dry and hot summer climate. In addition, this is the first exhaustive study on the survival of a yeast-BCA to T and RH controlled limiting conditions.

Under laboratory conditions, treatment with *C. sake* plus Fungicover showed high efficacy controlling *B. cinerea* infection of table grapes (75% severity reduction compared to untreated control) under very challenging artificial inoculation with selected virulent strains, including *B. cinerea*-II-*transposa* subpopulation strains. These strains are usually considered as the most aggressive type on grape berries (Martinez et al., 2005). Although FC alone reduced *B. cinerea* infection severity, *C. sake* significantly improved control compared to the additive itself and this difference remained in time, presenting severity under the 3% five days after inoculation and elevated reduction rates during the first seven days after inoculation.

When the BCA Full season treatment was applied in the vineyard, severity reduction (82% compared to untreated control), was similar to that observed in the laboratory experiment, confirming efficacy. Such a high efficacy level of *C. sake* and Fungicover field applications was obtained under conducive conditions for BBR. Under these humid Atlantic Bordeaux conditions, both incidence and severity reductions achieved by the Full season treatment were similar to those achieved in 2010 season in Lleida with a dry and hot summer climate (Calvo-Garrido et al., 2013), although the survival patterns were clearly different, as will be discussed below.

In 2010 season in Lleida, severity reductions were similar in Early season and Full season strategies (85% and 89%, respectively). However, in this experiment in Bordeaux, the Early season treatment showed a lower efficacy, although severity in the control was also high in 2010 (21.7 % severity; (Calvo-Garrido et al., 2013). Since *C. sake* populations remaining at harvest in Early season samples was notably high (Figure 4), the difference in severity reduction between the Full season and Early season treatments is unlikely to be linked to the effect of the *C. sake* population level. Thus, the effect of the two extra applications of FC after veraison in the Full season treatment could be the key point for accounting for the increased efficacy. Direct FC effect on *B. cinerea* has been shown clearly in our laboratory experiment (Figure 1) confirming previous published results (Calvo-Garrido et al., 2013). However, FC interaction with *L. botrana* development could also be hypothesized as playing an important role in BBR control in this study. First, it should be highlighted the well-established positive effect of *L. botrana* larvae (second and third summer generations) on BBR development (Fermaud and Giboulot, 1992; Fermaud and Lemenn, 1992). Thus, the very high *L. botrana* incidence observed in 2012 in untreated bunches (more than one larva of 3rd generation per bunch), should have highly promoted BBR epidemic development. A yeast effect on *L. botrana* larvae has not been tested, but is unlikely to occur. More presumably, we hypothesize that FC applications after veraison led to a decrease in *L. botrana* populations, which accounted for the reduction in BBR severity, explaining the significant differences between Full season and Early season treatments, independently of yeast populations. The effect on *L. botrana* may result from the presence of fatty acids (lauric, palmitic and stearic) as major FC ingredients, according to other studies reporting significant reduction of *L. botrana* oviposition in presence of stearic, palmitic and other long chain fatty acids (Gabel and Thiery, 1996; Thiery and Gabel, 1993; Thiery et al., 1995). The exact mode of action of FC on *L. botrana* requires further investigations but some hypotheses may be put forward such as direct toxicity on berry moth eggs or a repellent effect for adult females when they choose the site for egg deposition (Thiéry, Personal communication).

Overall, the presented results indicate that *C. sake* plus Fungicover treatments (both Early season and Full Season treatments) significantly reduced BBR severity, achieving chemical control reduction levels at commercial harvest, which could represent an effective alternative to synthetic fungicides under conducive Atlantic conditions. In addition, efficacy results were similar to those observed previously under dry Mediterranean conditions (Calvo-Garrido et al., 2013). This suggests an interesting inter-relationship between climate, *C. sake* survival and BBR: Atlantic Bordeaux conditions favour BBR development compared to Mediterranean Lleida conditions, but also favour *C. sake* survival leading to similar final disease reductions at harvest.

Survival of *C. sake* in field experiments carried out in Lleida region has been described as populations decreasing from one to four log (CFU g^{-1}) between spray applications (Calvo-Garrido et al., 2013; Cañamás et al., 2011). However, populations in our 2012 Bordeaux experiment showed no significant decrease between most of sprays. High and low field survival of yeast BCAs are reported in the literature, linked in some cases to the meteorological conditions during the experiments (Benbow and Sugar, 1999; Lima et al., 2003; Lima et al., 1997; Tian et al., 2004; Zahavi et al., 2000). The different survival pattern observed in Bordeaux and Lleida field experiments is consistent with the survival pattern evidenced in simulated climatic conditions experiment (Figure 8). Under the simulated conditions, a significant increase of around 0.5 log units was observed for samples exposed to BDX conditions compared with LDA samples, although T and RH variation between BDX and LDA regimes was reduced. Moreover, field conditions in dry Mediterranean-type regions include significant summer periods with air temperature over 35 °C and, in the vineyard, temperature is generally higher at the berry surface compared with air temperature (Pieri and Fermaud, 2005). These facts provide harsher conditions for BCA survival, since *C. sake* stops growing at 35 °C in NYDA medium (Teixidó et al., 1998c), whereas populations on berries also significantly decreased in 48 hours at 35 °C and 60% RH in climatic chambers (data not shown). The lethal effect of limiting conditions of T and RH on *C. sake* CPA-1 was corroborated, as shown in Figure 5, where populations rapidly decreased at 40 °C or 45°C. Moreover, population reductions due to limiting conditions at 40 °C were not significantly affected by a pre-exposure to BDX or LDA simulated conditions, although decrease was lower than after a period in optimal conditions, suggesting some adaptation. Thus, all our field and laboratory data illustrates and quantifies a lower *C. sake* survival under hot conditions, which may justify more frequent spray applications in warm climate vineyards and would provide opportunities to reduce applications in Atlantic-type regions, such as Bordeaux vineyards.

Great survival capacity on fruit surfaces of *C. sake* CPA-1 has been demonstrated since it is able to survive at nearly 0 °C temperatures in apple postharvest conditions (Teixidó et al., 1999, 1998a) and, in this study, the population was able to gradually decrease at 40 °C or 45 °C and constant 30% RH, observing measurable remaining populations after 48 or 72 hours (Figure 5). Yeasts are considered as potential BCAs due to their ability to cope with adverse environmental conditions and there are different examples of antagonist yeasts able to survive in a variety of pre- and/or post-harvest conditions (Benbow and Sugar, 1999; Ippolito et al., 2005; Karabulut et al., 2003; Zahavi et al., 2000). However, combination of high temperature and high RH was extremely harmful for *C. sake* cells. This result is in accordance with other studies that indicated a greater effect on yeast survival of RH compared with T (Lahlali et al., 2008; Teixidó et al., 1998b, 1998c), and also corroborates *in vitro* findings showing a dramatic *C. sake* population decrease in liquid medium at 40 °C and 45 °C (Cañamás et al., 2008). The combination of high T and high RH is not favourable for some fungal species, including BCAs (Agra et al., 2012; Cañamás et al., 2008) or fungal pathogens that, at the same temperature may be more affected in higher RH conditions (Teitel et al., 1989), providing opportunities for the control of fruit pathogens by curing treatments (Casals et al., 2010; Fallik, 2004). Low population survival under these conditions suggests reduced potential of *C. sake* for biological control in tropical regions or in association with post-harvest curing or hot water treatments.

The protective effect of the additive Fungicover on *C. sake* populations has been evidenced in previous field (Cañamás et al., 2011) and laboratory trials (Calvo-Garrido et al., Unpublished results). However, in this study, it has been demonstrated that FC did not protect directly *C. sake* from continued exposure under high temperature and low RH. Consequently, new hypotheses should be formulated to account for the observed FC effect on *C. sake* populations, notably in the field: i) the additive FC could protect a yeast antagonist such as *C. sake* CPA-1 by protecting from solar UV radiation, similarly to other BCA additives (Lahlali et al., 2011), and/or ii) the microenvironment created inside the FC biofilm on berries may protect against T and RH fluctuations, notably in sub-lethal ranges, as observed for additives protecting *C. oleophila* at 75% and 98% RH (Lahlali and Jijakli, 2009).

Lastly, the present survival study, under different limiting conditions, highlights the importance of the period immediately after *C. sake* application for its survival on grape berries. However, survival pattern of BCAs during the first hours after their application has been poorly studied, although effective establishment on a natural environment is considered to be crucial for subsequent efficacy (Jijakli, 2011).

In the present study, *C. sake* populations increased on berry surface in optimal conditions for at least 48 h (Figure 7a), while populations decreased less when there was an establishment period, in optimal conditions or simulated climatic regimes, before limiting conditions were applied (Figures 7b and 9). Furthermore, high sensitivity of the BCA was evidenced soon after application, since a very important decrease in population level was noticeable during the first 6 hours period, and then the decrease was relatively less marked until 24 hours post-application for most of the conditions tested (Figures 5, 6 and 9). These findings highlight the importance of choosing the right moment to perform field applications. To provide more favourable conditions for *C. sake* establishment during the first six hours post-application, a spray application 48 hours before the arrival of hot and dry conditions would minimise population decrease and evening applications may be desirable to avoid periods of high temperatures.

In conclusion, the present study under laboratory and field conditions corroborates the significant efficacy of treatments associating *C. sake* and Fungicover to control different virulent strains of *B. cinerea* as well as wild vineyard populations in the conducive Atlantic Bordeaux climate. In the field, the *C. sake* population monitoring and the newly-evidenced FC effect on *L. botrana* third-generation populations provided interesting information to better account for the field efficacy of the different treatment strategies to control BBR. In addition, survival studies, for the first time, have quantified the dynamics of *C. sake* populations under limiting and simulated climatic conditions, which lead us to draw possible strategies to maximise efficacy of field applications.

Acknowledgements

The authors are grateful to the University of Lleida for Carlos Calvo Garrido (CCG) PhD grant and the organic production research project, to the European Union for their financial support through the INTERREG REDBIO project and to the Catalan government for the CCG travel

grant. They are also grateful to the INRA UMR-Save for welcoming CCG in 2012 to carry out all the experiments presented in this paper by using the INRA research facilities in Bordeaux and to the Conseil Interprofessionnel du Vin de Bordeaux (CIVB) for its financial support to the project. They are indebted to UMR SAVE-Bordeaux staff: J.M. Liminana and J.M. Brustis for their technical support associated with climatic chambers and vineyard plot, respectively. Also to J. Jolivet, O. Bonnard, S. Capdevielle, J. Poidatz and L. Delbac for their valuable help with laboratory assays.

References

Agra, O., et al., 2012. Climate effect on pathogen - biocontrol agents interaction in the tomato - powdery mildew (*Oidium neolycopersici*) pathosystem. IOBC/WPRS Bulletin. 78, 233-237.

Andrews, J. H., 1992. Biological control in the phyllosphere. Annu. Rev. Phytopathol. 30, 603-635.

Benbow, J. M., Sugar, D., 1999. Fruit surface colonization and biological control of postharvest diseases of pear by preharvest yeast applications. Plant Dis. 83, 839-844.

Calvo-Garrido, C., et al., 2013. Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1. Plant Pathol. 62, 510-519.

Cañamás, T. P., et al., 2011. Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. Biol. Control. 56, 150-158.

Cañamás, T. P., et al., 2008. Impact of mild heat treatments on induction of thermotolerance in the biocontrol yeast *Candida sake* CPA-1 and viability after spray-drying. Journal of Applied Microbiology. 104, 767-775.

Casals, C., et al., 2010. Control of Monilinia spp. on stone fruit by curing treatments Part I. The effect of temperature, exposure time and relative humidity on curing efficacy. Postharvest Biology and Technology. 56, 19-25.

Deytieux-Belleau, C., et al., 2009. Grape berry skin features related to ontogenic resistance to *Botrytis cinerea*. Eur. J. Plant Pathol. 125, 551-563.

Droby, S., et al., 2009. Twenty years of postharvest biocontrol research: Is it time for a new paradigm? Postharvest Biol. Tec. 52, 137-145.

Elmer, P. A. G., Michailides, T. J., 2004. Epidemiology of *Botrytis cinerea* in orchard and vine crops. in: Elad, Y., et al., Eds.), *Botrytis*: Biology, Pathology and control. Kuwer academic publishers, Dordrecht, pp. 243-272.

Elmer, P. A. G., Reglinski, T., 2006. Biosuppression of *Botrytis cinerea* in grapes. Plant Pathol. 55, 155-177.

Fallik, E., 2004. Prestorage hot water treatments (immersion, rinsing and brushing). Postharvest Biol. Tec. 32, 125-134.

Fermaud, M., 1998. Cultivar susceptibility of grape berry clusters to larvae of *Lobesia botrana* (Lepidoptera : Tortricidae). J Econ. Entomol. 91, 974-980.

Fermaud, M., Gaunt, R. E., 1995. *Thrips obscuratus* as a potential vector of *Botrytis cinerea* in kiwifruit Mycol. Res. 99, 267-273.

Fermaud, M., Giboulot, A., 1992. Influence of *Lobesia botrana* larvae on field severity of *Botrytis* rot of grape berries. Plant Dis. 76, 404-409.

Fermaud, M., Lemenn, R., 1992. Transmission of *Botrytis cinerea* to grapes by grape berry moth larvae. Phytopathology. 82, 1393-1398.

Filonow, A. B., 1998. Role of competition for sugars by yeasts in the biocontrol of gray mold of apple. Biocontrol Sci. Techn. 8, 243-256.

Gabel, B., Thiery, D., 1996. Oviposition response of *Lobesia botrana* females to long-chain free fatty acids and esters from its eggs. J. Chem. Ecol. 22, 161-171.

Guetsky, R., et al., 2002. Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. Phytopathology. 92, 976-985.

Ippolito, A., Nigro, F., 2000. Impact of preharvest application of biological control agents on postharvest diseases of fresh fruits and vegetables. Crop Prot. 19, 715-723.

Ippolito, A., et al., 2005. Control of postharvest rots of sweet cherries by pre- and postharvest applications of *Aureobasidium pullulans* in combination with calcium chloride or sodium bicarbonate. Postharvest Biol. Tec. 36, 245-252.

Jijakli, M. H., 2011. *Pichia anomala* in biocontrol for apples: 20 years of fundamental research and practical applications. Antonie Van Leeuwenhoek. 99, 93-105.

Karabulut, O. A., et al., 2003. Near-harvest applications of *Metschnikowia fructicola*, ethanol, and sodium bicarbonate to control postharvest diseases of grape in central California. Plant Dis. 87, 1384-1389.

Kinay, P., Yildiz, M., 2008. The shelf life and effectiveness of granular formulations of *Metschnikowia pulcherrima* and *Pichia guilliermondii* yeast isolates that control postharvest decay of citrus fruit. Biol. Control. 45, 433-440.

Ky, I., et al., 2012. Assessment of grey mould (*Botrytis cinerea*) impact on phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages. Aust. J. Grape. Wine. R. 18, 215-226.

Lahlali, R., et al., 2011. Control of apple blue mold by the antagonistic yeast *Pichia anomala* strain K: screening of UV protectants for preharvest application. Plant Dis. 95, 311-316.

Lahlali, R., Jijakli, M. H., 2009. Enhancement of the biocontrol agent *Candida oleophila* (strain O) survival and control efficiency under extreme conditions of water activity and relative humidity. Biol. Control. 51, 403-408.

Lahlali, R., et al., 2009. Assessment of *Pichia anomala* (strain K) efficacy against blue mould of apples when applied pre- or post-harvest under laboratory conditions and in orchard trials. Eur. J. Plant Pathol. 123, 37-45.

Lahlali, R., et al., 2008. A Box-Behnken design for predicting the combined effects of relative humidity and temperature on antagonistic yeast population density at the surface of apples. Int. J. Food Microb. 122, 100-108.

Lima, G., et al., 2002. Survival and activity of biocontrol yeasts against powdery mildew of cucurbits in the field. Bulletin OILB/SROP. 25, 187-190.

Lima, G., et al., 2003. Integrated control of apple postharvest pathogens and survival of biocontrol yeasts in semi-commercial conditions. Eur. J. Plant Pathol. 109, 341-349.

Lima, G., et al., 1997. Biological control of grey mould of stored table grapes by pre-harvest applications of *Aureobasidium pullulans* and *Candida oleophila*. Difesa delle Piante. 20, 21-28.

Magan, N., 2001. Physiological approaches to improving the ecological fitness of fungal biocontrol agents. in: Butt, T. M., et al., (Eds.), Fungi as biocontrol agents: progress, problems and potential. CABI Publishing, pp. 239-251.

Manso, T., Nunes, C., 2011. *Metschnikowia andauensis* as a new biocontrol agent of fruit postharvest diseases. Postharvest Biol. Tec. 61, 64-71.

Martinez, F., et al., 2003. Phenotypic differences between *vacuma* and *transposa* subpopulations of *Botrytis cinerea*. Eur. J. Plant Pathol. 109, 479-488.

Martinez, F., et al., 2008. New PCR primers applied to characterize distribution of *Botrytis cinerea* populations in French vineyards. Vitis. 47, 217-226.

Martinez, F., et al., 2005. The role of saprotrophy and virulence in the population dynamics of *Botrytis cinerea* in vineyards. Phytopathology. 95, 692-700.

Meng, X., Tian, S., 2009. Effects of preharvest application of antagonistic yeast combined with chitosan on decay and quality of harvested table grape fruit. J. Sci. Food Agric. 89, 1838-1842.

Nally, M. C., et al., 2012. Biocontrol of *Botrytis cinerea* in table grapes by non-pathogenic indigenous *Saccharomyces cerevisiae* yeasts isolated from viticultural environments in Argentina. Postharvest Biol. Tec. 64, 40-48.

Nicot, P. C., 2011. Classical and Augmentative Biological Control Against Diseases and Pests: Critical Status Analysis and Review of Factors Influencing Their Success. IOBC/WPRS.

Parry, F., et al., 2011. Developing new biologically-based products for control of botrytis bunch rot. Part 2: Developing a new biologically-based product for late-season botrytis control: The BCA-L1 story. Wine Vitic. J. 26, 73-78.

Pieri, P., Fermaud, M., 2005. Effects of defoliation on temperature and wetness of grapevine berries. Proceedings of the Seventh International Symposium on Grapevine Physiology and Biotechnology. 109-116.

Teitel, D. C., et al., 1989. The use of heat-treatments to extend the shelf-life of Galia melons. J. Hortic. Sci. 64, 367-372.

Teixidó, N., et al., 2010. Preharvest strategies to control postharvest diseases in fruits. in: Prusky, D., Gullino, M. L., Eds.), Postharvest Pathology. Springer, pp. 89-106.

Teixidó, N., et al., 2001. Improving control of green and blue molds of oranges by combining *Pantoea agglomerans* (CPA-2) and sodium bicarbonate. Eur. J. Plant Pathol. 107, 685-694.

Teixidó, N., et al., 1999. Efficacy of preharvest and postharvest *Candida sake* biocontrol treatments to prevent blue mould on apples during cold storage. Int. J. Food Microbiol. 50, 203-210.

Teixidó, N., et al., 1998a. Effect of the antagonist *Candida sake* on apple surface microflora during cold and ambient (shelf life) storage. Eur. J. Plant Pathol. 104, 387-398.

Teixidó, N., et al., 1998b. Control of blue mold of apples by preharvest application of *Candida sake* grown in media with different water activity. Phytopatology. 88, 960-964.

Teixidó, N., et al., 1998c. Ecophysiological responses of the biocontrol yeast *Candida sake* to water, temperature and pH stress. J. Appl. Microbiol. 84, 192-200.

Thiery, D., Gabel, B., 1993. Inter-specific avoidance of egg-associated semiochemicals in 4 tortricids. Experientia. 49, 998-1001.

Thiery, D., et al., 1995. Egg dispersion in codling moth: Influence of egg extract and of its fatty acid constituents. J. Chem. Ecol. 21, 2015-2026.

Tian, S. P., et al., 2004. Survival of antagonistic yeasts under field conditions and their biocontrol ability against postharvest diseases of sweet cherry. Postharvest Biol. Tec. 33, 327-331.

Valdés-Gómez, H., et al., 2008. Grey mould incidence is reduced on grapevines with lower vegetative and reproductive growth. Crop Prot. 27, 1174-1186.

Viñas, I., et al., 1998. Biological control of major postharvest pathogens on apple with *Candida sake*. Int. J. Food Microbiol. 40, 9-16.

Zahavi, T., et al., 2000. Biological control of *Botrytis, Aspergillus* and *Rhizopus* rots on table and wine grapes in Israel. Postharvest Biol. Tec. 20, 115-124.

CAPÍTULO 7

Survival of the biological control agent *Candida sake* CPA-1 on grapes under the influence of abiotic factors

C. Calvo-Garrido, I. Viñas, J. Usall, M. Rodríguez-Romera, M.C.Ramos and N. Teixidó

Enviado a: ***BioControl***

Survival of the biological control agent *Candida sake* CPA-1 on grapes under the influence of abiotic factors

Authors

Calvo-Garrido, C[1]; Viñas, I[1]; Usall, J[2]; Rodríguez-Romera, M[3]; Ramos MC[4] and Teixidó, N[2]

[1] *Food Technology Department, University of Lleida, XaRTA-Postharvest, Agrotecnio Center, Rovira Roure 191, 25198 - Lleida, Catalonia, Spain*

[2] *IRTA, XaRTA-Postharvest, Rovira Roure 191, 25198 - Lleida, Catalonia, Spain*

[3] *Laboratorio de Fitopatología- EEA INTA Mendoza. San Martín 3853. Mayor Drummond. Luján de Cuyo. Mendoza. Argentina (M5507EVY)*

[4] *Environment & Soil Science Department, University of Lleida, Agrotecnio Center, Rovira Roure 191, 25198 - Lleida, Catalonia, Spain.*

Abstract

Reliability of preharvest applications of biological control agents (BCAs) to control fruit pathogens is highly dependent on the survival of the selected organism. Present study describes population dynamics of the yeast-BCA *Candida sake* CPA-1 on grape berries under the effect of abiotic factors such as temperature, relative humidity, sunlight and rainfall.

The addition of the food additive Fungicover® significantly increased *C. sake* multiplication under optimal growth conditions and improved survival under fluctuating abiotic factors. After field applications, significant differences in populations on grape bunches exposed or covered by fine foliage were detected.

Simulated rainfall washed off *C. sake* populations by 0.6 to 0.9 log units after 20 mm of rain volume, whereas an establishment time on berries, prior to rain event, significantly increased persistence independently of the effect of rain intensity. Significance of population survival and practical outcomes of the results to improve efficacy of *C. sake* treatments are discussed.

Keywords

extreme conditions, fatty acids, rainfall, sunlight , vineyard, *Vitis vinifera*

Introduction

The use of selected microorganisms to control fruit pathogens has been widely studied in the past years showing promising results of disease reduction in pre- and post-harvest applications, with several formulated products already available in the market, although achieved control showed high variability in some cases (Glare *et al*., 2012, Teixidó *et al*., 2011).

In particular, when mode of action of fungal antagonists relies in spatial and nutrient competition, efficacy of BCA treatments is strongly determined by maintaining a stable BCA population at high concentration, identifying a density threshold of 10^4 CFU cm^{-2} to ensure pathogen suppression (Andrews, 1992, De Clercq *et al.*, 2003). Therefore, the study of populations on the host plant is crucial to understand subsequent efficacy of BCA treatments and develop strategies to improve their performance. Populations of different BCAs have been reported to maintain high densities on fruit surfaces during long periods of postharvest storage (Cañamás *et al*., 2008b, Ippolito *et al*., 2005, Nunes *et al*., 2001, Qin *et al*., 2006) where conditions of temperature (T), relative humidity (RH) and O_2/CO_2 concentrations are generally controlled and stable, providing a favourable environment for population survival of the selected BCA.

However, when dealing with preharvest applications, populations on plant surfaces are exposed to a range of fluctuating abiotic factors, particularly water availability, T, length of dew periods, microclimate, canopy type and rainfall events (Magan, 2001). These fluctuations may affect BCA survival, as frequently observed in studies monitoring population dynamics after field applications (Benbow & Sugar, 1999, Cañamás *et al*., 2008c, Lima *et al*., 2002, Longa *et al*., 2007), including the yeast *C. sake* on grapes (Calvo-Garrido *et al*., 2013, Cañamás *et al*., 2011). Interesting research has evaluated population of fungal BCAs under the effect of abiotic factors such as T and water availability (Lahlali *et al*., 2008, Nicot *et al*., 2002, Teixidó *et al*., 1998) or sunlight and UV radiation (Behle *et al*., 1997, Köhl *et al*., 2002, Lahlali *et al*., 2011, Morandi *et al*., 2007), as well as the protective effect against these factors provided by different additives or adjuvants for BCA application (An *et al*., 2013, Cañamás *et al*., 2008a, Lahlali & Jijakli, 2009). However, more information is needed to understand how much abiotic factors reduce populations and the progression of the decrease in time, in order to predict hypothetic population losses and hence design application programs to achieve high treatment efficacy.

In addition, the effect of other abiotic factors such as rainfall on BCAs has been scarcely studied. As far as the authors know, only two research works have specifically evaluated the effect of rainfall on microorganisms for biological control, testing residual efficacy of the bacteria *Bacillus thuringiensis* var. Kurstakii against *Trichoplusia ni* (Hubner) (Behle *et al.*, 1997) and the persistence of the entomopathogenic fungi *Beauveria bassiana* on alfalfa and wheat crops (Inglis *et al.*, 1995). However, rainfall may be responsible of treatment wash-off after application and is considered to be a main factor influencing efficacy of treatments with copper preparations (Hunsche *et al*., 2011, Molot & Gaimon, 2004), synthetic fungicides (Cabras *et al*., 2001, Hunsche *et al*., 2007) and insect BCAs against pests (Fink & Volkl, 1995, Norris *et al*., 2002). Some factors influencing wash off of agrochemicals due to rainfall are rain volume, rain intensity, time between application and rainfall event and type of crop plant (Cabras *et al*., 2001, Hunsche *et al*., 2007).

The present study aimed (1) to evaluate population dynamics of the yeast-BCA *C. sake* CPA-1 exposed to main abiotic factors constraining survival in field conditions, such as T, RH and sunlight; (2) to investigate the protective effect of the additive Fungicover® (FC) on *C. sake* survival and (3) to study the effect of simulated rainfall with different rain intensity, rain volume and time length between application and rain events on *C. sake* persistence.

Materials and methods

Plant material and vineyard characteristics

Grape berries used in the survival experiments were sampled from a commercial organic vineyard certified by the Catalan committee for organic agriculture production (CCPAE) and located in the Designation of Origin Costers del Segre (Lleida, Catalonia, Spain). The cultivar used was Macabeu and vines were trained in the typical *goblet* system used by growers in the region. Sound grape bunches were sampled from untreated vines using sterile clippers and individually introduced in paper bags. Then transported in an ice-box to the laboratory and stored at 4 °C prior to the assay. Manipulation of bunches to obtain the grape clusters used in the assays was always carried out with sterile clippers and gloves. Experimental design of the field experiment carried out in the same vineyard is described in a later section.

C. sake *cell production and formulation*

The BCA *C. sake* CPA-1 was originally isolated from apples in the IRTA Lleida centre and was deposited at the "Colección Española de Cultivos Tipo" (CECT-10817) in the University of Valencia, Burjassot, Spain. Stock cultures were stored on Nutrient Yeast Dextrose Agar medium (NYDA: nutrient broth, 8 g l^{-1}; dextrose, 10 g l^{-1}; and agar, 15 g l^{-1}) at 4 °C. When required, *C. sake* CPA-1 was subcultured onto NYDA plates at 25 °C. Then, subcultured cells suspended on potassium phosphate buffer (PhB; KH_2PO_4 0.2 mol l^{-1}, 70 ml; K_2HPO_4 0.2 mol l^{-1}, 30 ml and deionised water, 300 ml) were added as inoculum starter to 5 l of molasses-based medium (MB; cane molasses 40 g l^{-1}, urea 1.2 g l^{-1}; water activity a_w = 0.996) adjusting initial concentration to 1 x 10^6 CFU ml^{-1}. Cell pellets were obtained by centrifugation at 6831 g for 10 min at 10 °C after 40 h of liquid fermentation at 25 °C, 400 rpm agitation speed and 150 l h^{-1} aeration level. Resuspended pellets were then formulated in an isotonic solution adjusting water potential with trehalose as described previously (Abadias *et al.*, 2003).

C. sake was applied on grape berries with the additive Fungicover (Biodúrcal S.L., Spain) in all the experiments. Fungicover was included because this additive has shown to aid field survival of *C. sake* (Cañamás *et al.*, 2011).

Survival of C. sake *populations on grape berries under controlled or outdoor conditions*

The dynamics of *C. sake* populations on berry surface were monitored to compare the population numbers on berries incubated in controlled optimal conditions for *C. sake* growth

and populations exposed to outdoor summer conditions. In addition, the effect of FC on *C. sake* populations was evaluated by applying *C. sake* with different concentrations of FC prior to the exposure to controlled or outdoor conditions.

Clusters of five grape berries were placed onto plastic trays with plastic grids and sprayed with *C. sake* $5x10^7$ CFU ml^{-1} alone (CS), mixed with FC at 25 g l^{-1} (CS+FC25) or 50 g l^{-1} (CS+FC50), using a motorised backsprayer (model WJR2225; Honda Motor Company Ltd, Germany) and let dry for a two hours period at room temperature. Then, a set of samples were incubated in controlled conditions, by introducing the trays in a climatic chamber at constant 25 °C and 85% RH. The rest of samples were placed onto trays and transported to an outdoor flat-roof at IRTA-Lleida research centre, exposed to the limiting conditions of the Mediterranean climate summer and the subsequent T , RH and sunlight fluctuations. In order to simulate the partial shading provided by foliage in the vineyard, all the trays were covered with a shade-cloth placed 2 m above the trays, which softened the effect of direct sunlight during approximately five hours along the day.

Each treatment consisted of four replicates and each replicate consisted of four treated clusters of five berries each. Populations on grape berry surface were recovered after 0, 24 and 72 hours in controlled conditions or exposed to outdoor conditions. The 20 berries per replicate were individually cut leaving the pedicel attached and introduced in 250-ml Erlenmeyer flasks containing 50 ml of PhB. Then shaken for 20 min at 150 rpm on a rotary shaker and sonicated for 10 min in an ultrasonic bath (JP Selecta S.L., Spain). After serial dilutions of the washing solution, 100 µL aliquots were plated onto NYDA plates (NYDA: nutrient broth, 8 g l^{-1}; yeast extract, 5 g l^{-1}; dextrose, 10 g l^{-1}; and agar, 15 g l^{-1}) supplemented with streptomycin sulphate (0.5 g l^{-1}) to avoid bacterial growth. Duplicate plates were incubated in the dark at 25 °C and colonies were visually recognised and counted, based on their morphological characteristics, after 48 hours. Data was collected as CFU ml^{-1} and expressed as CFU g^{-1} of tissue sample. The experiment in outdoor conditions was repeated twice in two different weeks during the season (16/08/2010 and 23/08/2010), whereas the incubation in controlled conditions was carried out once. The CFU g^{-1} data of the *C. sake* populations reduction were finally expressed as Log ($N\ N_0^{-1}$), where N = Populations in the sample after exposure to the different conditions tested (CFU g^{-1}) and N_0 = Populations before exposure. Meteorological data (T, RH and rainfall) of the three day periods of both replicate experiments were collected by a weather station of the Catalan Government meteorological service, placed five kilometres away from IRTA-Lleida research center.

Effect of Fungicover on C. sake *populations in phosphate buffer*

The population dynamics of *C. sake* cells incubated in PhB with different concentrations of Fungicover was evaluated in order to determinate the effect of FC composition on *C. sake* growth.

For that purpose, 50 ml of PhB were introduced in 250 ml Erlenmeyer flasks and FC was added adjusting concentrations to 0, 25 or 50 g l^{-1} (CS, CS+FC25 and CS+FC50 treatments, respectively). Three flasks per treatment were prepared and considered as replicates. Then, the Erlenmeyer

flasks were incubated at 25 °C in a rotary shaker incubator (Certomat® BS-1, Sartorius AG, Germany) at 150 rpm. After 0, 24, 28 and 48 hours of incubation, 0.5 ml samples were obtained from each replicate and, after serial dilutions, 100 µL aliquots were plated in NYDA medium. Replicate plates were incubated for 48 hours prior to colony counts and results were expressed as CFU ml^{-1}.

Effect of leaf removal on C. sake *field survival on grape bunches*

Two rows of ten vines each were selected in the experimental vineyard, considering the first and last vines as buffer vines. In one of the rows, vines were untouched and four bunches per vine were labelled, choosing bunches that were approximately 90% covered from sunlight by foliage (COV). On the other row, vines were trimmed, removing leaves to achieve an approximately 10% coverage of grape bunches (EXP), and four bunches per vine were labelled too. Thus, the final number of bunches in each treatment (COV or EXP) was 32, divided in four replicates that consisted of eight bunches in two vines.

The mixture of *C. sake* at $5x10^7$ CFU ml^{-1} plus FC at 50 g l^{-1} was applied to the labelled bunches until runoff with a motorised backsprayer (model WJR2225; Honda Motor Company Ltd, Germany). When treatment mixture on bunches was dry, initial *C. sake* populations were evaluated. Populations on berry surface were also evaluated after 1, 3, 4, 7 and 14 days after the first sampling. Twenty berries were randomly sampled from the eight bunches in each replicate, placed in sterile plastic tubes and stored in an ice-box prior to sample processing in the laboratory. Population recovery from berry surface was carried out as described above. The experiment was repeated twice in two different weeks and using two different parts of the same vineyard (11/07/2011 to 25/07/2011; 05/09/2011 to 19/09/2011). In both replicate experiments, measurements of T, RH and rainfall and at hourly intervals were collected using a weather station (Decagon Services Inc., USA) placed on the experimental plot.

Persistence of C. sake *populations on berry surface under simulated rainfall*

To evaluate the decrease of *C. sake* populations on berry surface due to rainfall, grape clusters treated with *C. sake* plus FC were exposed to different rain volumes and intensities prior to population recovery.

Four clusters of approximately 20 berries each were cut to form one replicate, whereas each treatment consisted of four replicates. The four replicates of each treatment were sprayed with *C. sake* ($5x10^7$ CFU ml^{-1}) plus FC (50 g l^{-1}) using a motorised backsprayer (model WJR2225; Honda Motor Company Ltd, Germany) and dried for two hours at room temperature. Then, clusters were directly introduced in a rain simulator or incubated in a climatic chamber at 25 °C and 85% RH during 24 hours, 72 hours or 7 days prior to exposure to simulated rain. This incubation time provided an establishment period in favourable conditions for *C. sake* applied on berries, which may affect the population loss due to a later rainfall event.

Rainfall was simulated using a rainfall simulator consisting of a metallic box 100*50*20 cm with a drop generator system at the bottom. The droppers (50 mm separation among them) generated 2.5 mm diameter drops which fell freely 1.5 m above the tray in which the samples were located. In addition, a moving fan was placed in front of the rain curtain in order to interfere with drop fall and avoid the continuous impact of rain drops on the same cluster parts. Grape clusters were exposed to three rain intensities: 60, 100 and 150 mm h^{-1}, with an overall 5% variation of intensity. The intensity was regulated maintaining a constant water layer above the droppers, while uniformity of rain intensity was measured before and after each rain event, by measuring intensity in two graduated beakers placed under the simulator. For each rain intensity and establishment time, the *C. sake* populations were recovered after 0, 20, 60 and 120 mm of rain volume. Five berries from each of the four clusters were cut leaving the pedicel attached, obtaining 20 berries per replicate, which were introduced in Erlenmeyer flasks with 50 ml of PhB for population recovery as described in previous sections.

Results of the *C. sake* populations reduction were finally expressed, for each rain depth sample, as Log ($N\ N_0^{-1}$), where N = Populations in the sample exposed to rainfall (CFU g^{-1}) and N_0 = Populations before exposure to rainfall (CFU g^{-1}).

Statistical analysis

Statistical analysis was carried out with the JMP8 Software (SAS Institute Inc., USA). Original CFU g^{-1} data were transformed to Log_{10} (x) prior to ANOVA to improve homogeneity of variances. Significant treatment differences were determined using Tukey test (P = 0.05) in all samples.

Results

Survival of C. sake *populations on grape berries under controlled or outdoor conditions*

Overall, significant differences were observed between *C. sake* populations on berry clusters incubated in controlled optimal conditions and those exposed to abiotic factors. Thus, data analysis was carried out for both conditions separately for a better understanding of treatment effects.

Results of the *C. sake* populations recovered from berries that had been incubated under controlled optimal conditions are summarised in Fig. 1a. Populations in the three evaluated treatments were similar at 0 h. After 24 h incubation, populations significantly increased in the CS+FC25 and the CS+FC50 treatments more than 0.6 log units, whereas no growth was observed in the CS treatment samples. After 72 h of incubation, populations (Mean ± SE) in CS samples significantly increased 0.3 ± 0.08 log units compared to the initial concentration on berry surface.

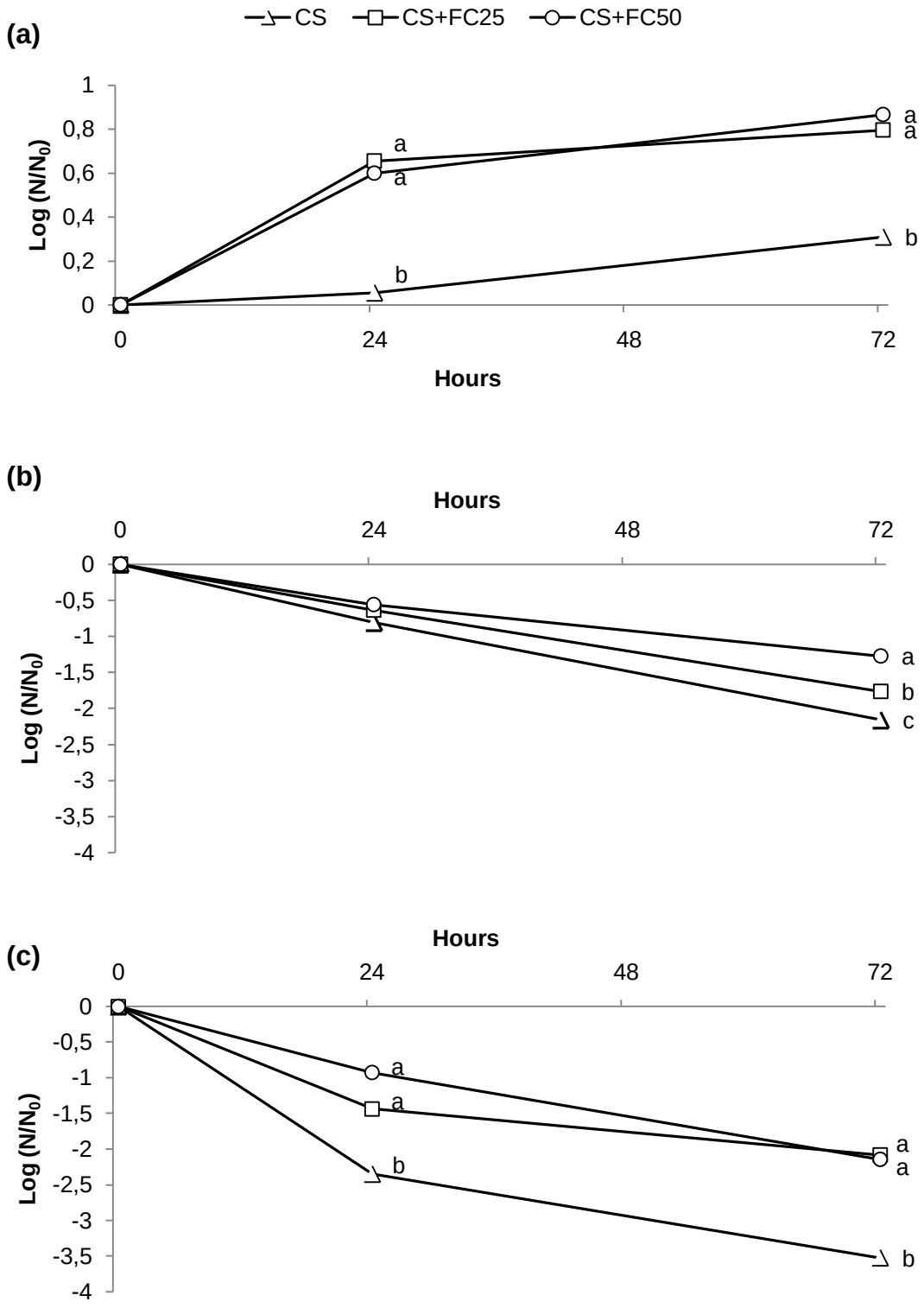


Figure 1 Population dynamics of *C. sake* CPA-1 on Macabeu grape berry clusters under a) Incubation at 25 ºC and 85% RH; b) Exposure to summer outdoor conditions (first replicate experiment; 16/08/2010); c) Exposure to summer outdoor conditions (second replicate experiment; 23/08/2010). *C. sake* was applied at a $5x10^7$ CFU g^{-1} rate alone (CS), with Fungicover® at 25 g l^{-1} (CS+FC25) or with Fungicover® at 50 g l^{-1} (CS+FC50). Values are means of four replicates. Mean values connected by the same letter are not significantly different (P= 0.05) according to Tukey test.

Mean populations also increased in the treatments including FC, arriving to 0.8 ± 0.07 and 0.9 ± 0.15 log units more than the initial populations (CS+FC25 and the CS+FC50, respectively). Populations at 24 h and 72 h of incubation in treatments with FC were similar between them, while both were significantly different ($P < 0.05$) from populations in the CS treatment.

The Analysis of variance of the two replicate experiments evaluating *C. sake* exposed to summer outdoor conditions evidenced significantly lower ($P < 0.05$) overall populations in the second experiment samples and also significant interactions ($P < 0.05$) between the treatment, the sampling time and the experiment replicate. Therefore, population dynamics of *C. sake* in the two replicate experiments could not be pooled and are represented separately in Fig. 1b, c.

In the first experiment (Fig. 1.b), 24 h after the start of the exposure, the *C. sake* populations significantly decreased ($P < 0.05$) in the three treatments between -0.6 ± 0.13 and -0.8 ± 0.07 log units (CS+FC50 and CS, respectively). Nonetheless, no significant differences ($P < 0.05$) were observed among treatments. Then, after 72 hours, populations significantly decreased in the three treatments as well. Final reduction, compared to populations at 0 h, was -1.3 ± 0.22, -1.8 ± 0.24 and -2.2 ± 0.04 log units (CS+FC50, CS+FC25 and CS, respectively) and significant differences were detected among all treatments.

In the second experiment (Fig. 1c), populations were significantly reduced by -0.9 ± 0.18 (CS+FC50) to -2.3 ± 0.17 (CS) log units in the first 24 h of exposure to outdoor conditions and the CS+FC50 and CS+FC25 treatments were significantly different ($P < 0.05$) from the CS treatment. At 72 h, reductions were much higher than in the first replicate experiment and ranged from -2.1 ± 0.19 and -2.1 ± 0.28 (CS+FC25 and CS+FC50, respectively) to -3.5 ± 0.12 (CS) log units compared to initial populations. Contrarily to the first experiment, the CS+FC50 and CS+FC25 treatments were similar between them and significantly different ($P < 0.05$) from the CS treatment at 72 h.

During the 72 hours of exposure in the first experiment, mean (± SE) values of T and RH were 23.4 ± 1.14 °C and 60.2 ± 4.64 %, respectively. In the second replicate experiment, mean T and RH values were 27.1 ± 0.44 °C and 51.7 ± 2.01 %.

Effect of Fungicover on C. sake *populations in phosphate buffer*

After 24 h of incubation at 25 ^{0}C, concentration of *C. sake* cells significantly increased for more than one log unit ($P < 0.05$) compared to the initial concentration in the three treatments (Fig. 2). However, no significant differences ($P < 0.05$) were detected among treatments.

After 28 hours, significant differences were observed ($P < 0.05$) between populations incubated in PhB (CS) and the populations incubated in PhB with the addition of FC (CS+FC25 and CS+FC50).

Finally, after 48 h incubation, populations continued increasing and final CFU ml^{-1} were 5.6 ± 0.08 (CS), 6.5 ± 0.01 (CS+FC25) and 6.8 ± 0.05 (CS+FC50) log units. Similarly to the 28 h sample, treatments with FC (CS+FC25 and CS+FC50) presented significantly higher populations than the treatment with *C. sake* alone (CS).

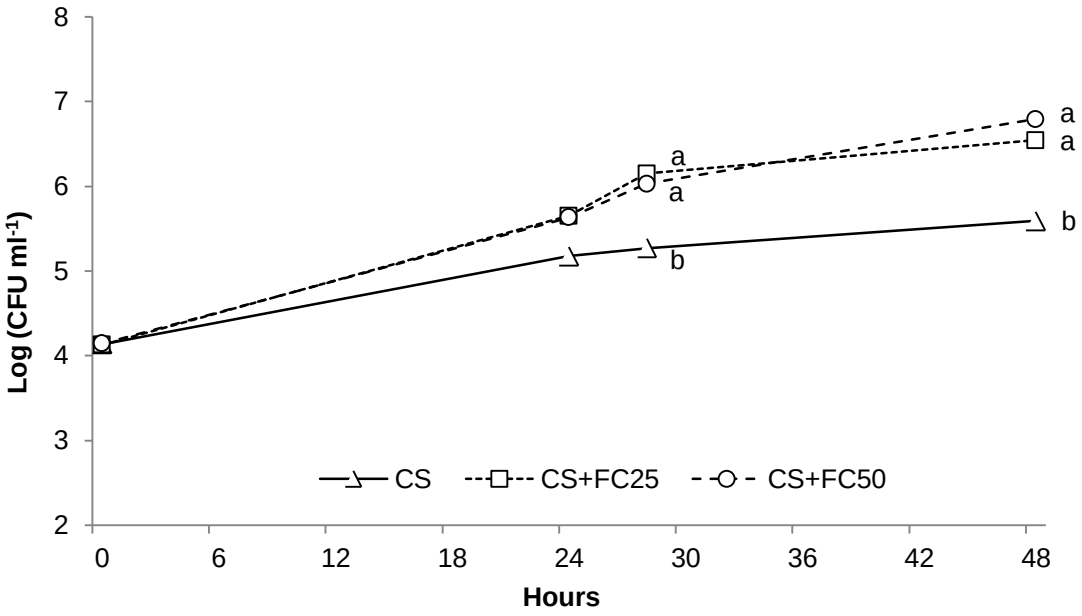


Figure 2 Population dynamics of *C. sake* CPA-1 cells incubated in phosphate buffer amended with Fungicover. The additive Fungicover was added at a concentration of 0 g l^{-1} (CS), 25 g l^{-1} (CS+FC25), and 50 g l^{-1} (CS+FC50). Erlenmeyer flasks containing the different mixtures were inoculated with formulated *C. sake* adjusting final concentration to $1x10^4$ CFU ml^{-1} and incubated at 25 ºC on a rotary shaker in the dark. Population counts were carried out after serial dilutions and plating of aliquots of the incubated solutions. Values are means of three replicates. Mean values connected by the same letter are not significantly different ($P = 0.05$) according to Tukey test.

Effect of leaf removal on C. sake *field survival on grape bunches*

Since significant interaction was observed between the treatment effects and the replicate experiment, data could not be pooled for the subsequent analysis (Fig. 3a, b). The analysis of the interaction showed that in the first replicate experiment, mean populations on exposed bunches were significantly lower ($P < 0.05$) than in the second experiment, whereas mean populations in samples from covered bunches (COV) were similar in both experiments.

In both experiments, *C. sake* populations on grape berries decreased remarkably in the first 24 hours after field application. Populations decreased significantly in both treatments. However, populations decreased more in EXP samples, and significant differences ($P < 0.05$) were observed between covered and exposed bunches after 24 h in both experiments. In this 24 h period, populations decreased (Mean ± SE) -0.7 ± 0.03 (COV) and -1.5± 0.31 (EXP) log units in the first assay, whereas in the second assay populations decreased -0.6 ± 0.08 (COV) and -1.0 ± 0.07 (EXP) log units, compared to initial concentrations.

In the first assay (Fig. 3a), after decreasing in the first 24 hours, populations on both covered and exposed bunches did not significantly decrease in the period between 1 day and 7 days after treatment application. The differences ($P < 0.05$) between COV and EXP samples were significant and ranged from 0.8 ± 0.23 to 1.3 ± 0.28 log units in the different sample times.

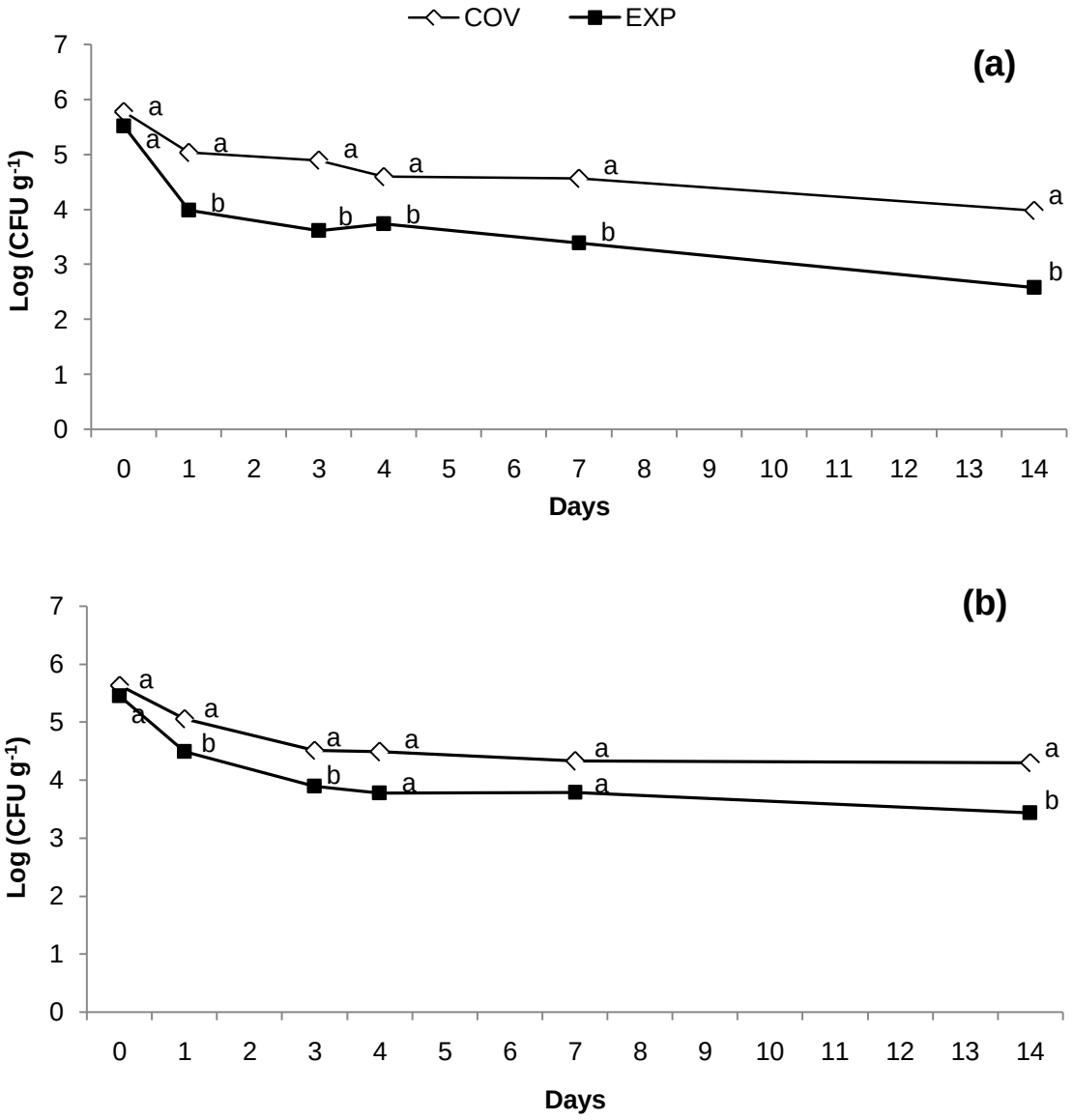


Figure 3 Effect of leaf removal on the population dynamics of *C. sake* after field application on Macabeu grape bunches. a) First replicate experiment and b) second replicate experiment. *C. sake* ($5x10^7$ CFU g^{-1}) was applied with Fungicover at 50 g l^{-1} on selected bunches, which were covered approximately 90% (COV) or 10% (EXP) by vine foliage. Values are means of four replicates per treatment. Mean values connected by the same letter at the same sample time are not significantly different ($P = 0.05$) according to Tukey test.

Between 7 and 14 days after the application, populations decreased significantly in both treatments, and final populations on berries were 4.0 ± 0.10 CFU g^{-1} (COV) and 2.6 ± 0.39 CFU g^{-1} (EXP).

In contrast, in the second assay (Fig. 3b) populations decreased gradually in both COV and EXP treatments until sampling at 3 days after the application. From that point on, populations did not decrease significantly. Difference between treatment samples ranged from 0.6 ± 0.08 to 0.9 ± 0.14 log units and were significant ($P < 0.05$) in every sampling time except in the 4 days and 7 days samples, observing final remaining populations of 4.3 ± 0.15 CFU g^{-1} (COV) and 3.4 ± 0.14 CFU g^{-1} (EXP), 14 days after field application.

Mean (± SE) T and RH observed during the first replicate experiments were 20.9 ± 0.22 °C and 56.6 ± 0.93 %, whereas in the second experiment were 21.5 ± 0.30 °C and 66.3 ± 1.09 %. The mean temperature during the first 24 hours after the application was approximately 5 °C higher in the first experiment.

Persistence of C. sake *populations on berry surface under simulated rainfall*

The *C. sake* populations on grape berries were evaluated after the exposure to different quantities of simulated rain, different rain intensities and different BCA establishment times prior to exposure. Since the effect of intensity was different depending on the establishment times, results of the assays carried out with the four different establishment times are presented separately (Fig. 4a, b, c, d).

The effect of rain intensity was not overall significant ($P < 0.05$). However, when the establishment time was 0 hours (Fig. 4a), the mean population reduction of three rain volumes caused by the lower rain intensity (-0.6 ± 0.07 log units; 60 mm h^{-1}) was significantly lower than the reduction caused by simulated rain of 100 or 150 mm h^{-1} (-0.9 ± 0.05 and -1.0 ± 0.07 log units, respectively). When the samples were incubated providing a establishment period prior to rainfall exposure (24 hours, 72 hours, 7 days), no significant differences were detected among intensities.

When all intensities and rain volumes were analysed together (60, 100, 150 mm h^{-1}; 20, 60, 120 mm), overall reduction when the establishment time was 0h (-0.8 ± 0.06 log units) was significantly higher, compared to samples with a previous establishment period. When there was a 24 hours period, the reduction was significantly lower (-0.6 log ± 0.10 units), although it was still significantly different ($P < 0.05$) from reductions in samples with establishment periods of 72 hours and 7 days (-0.4 ± 0.09 and -0.5 ± 0.08, respectively).

In addition, the occurrence of the establishment period also significantly affected the dynamics of *C. sake* population reductions after different rain volumes. When establishment time was 0 h (Fig. 4a), populations rapidly decreased after the exposure to the first 20 mm of rain and then maintained similar levels, without significant differences ($P < 0.05$) among the 20, 60 and 120 mm samples. Nonetheless, when there was an establishment time (24 hours, 72 hours or 7 days), reduction after the first 20 mm was lower and populations continued decreasing gradually, evidencing significant differences between the populations in the 20 mm and the 120 mm samples.

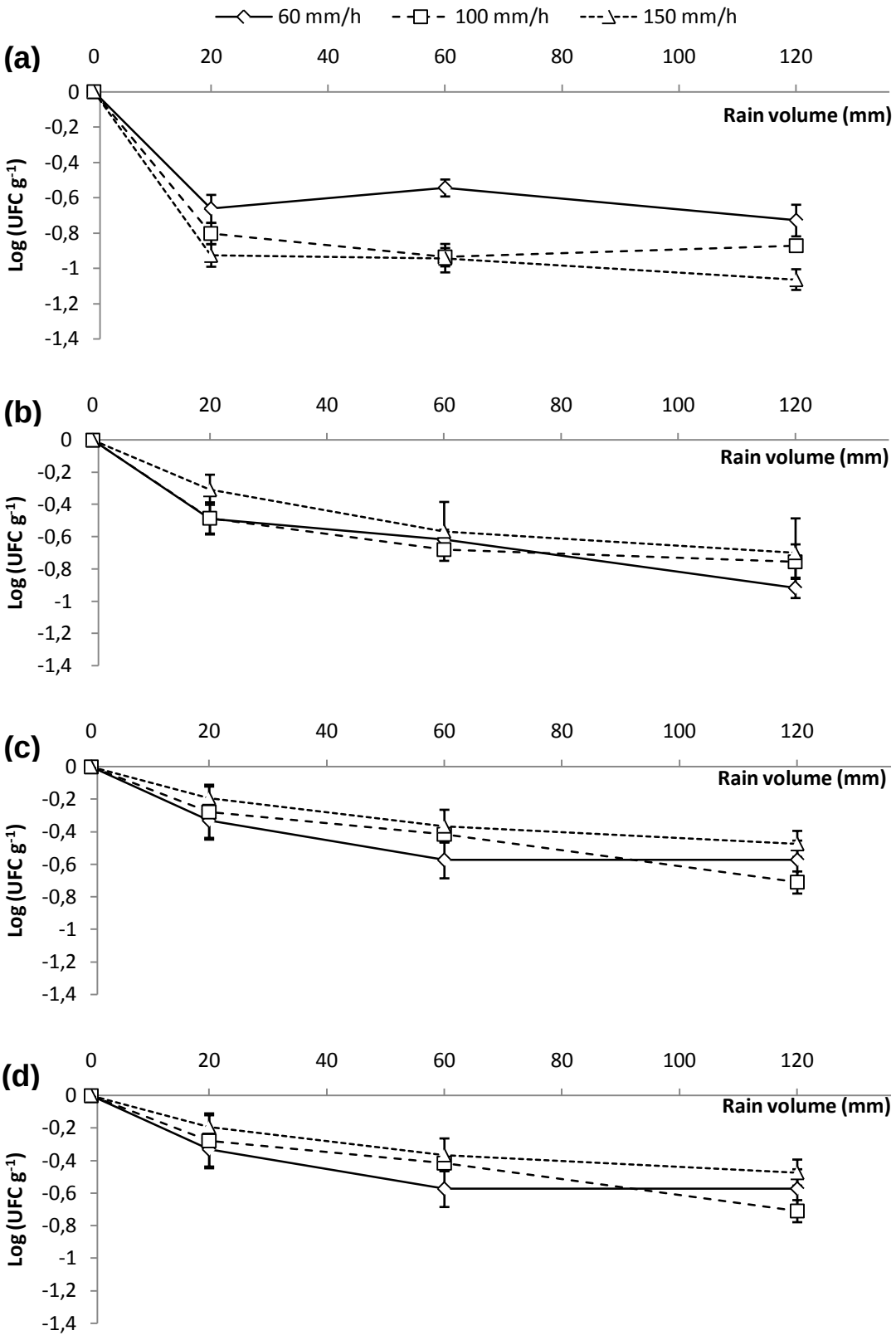


Figure 4 Population dynamics of *Candida sake* CPA-1 on Macabeu grape berry clusters exposed to simulated rainfall. *C. sake* ($5x10^7$ CFU g^{-1}) was applied with Fungicover at 50 g l^{-1} on grape clusters of 20 berries. Then, clusters were incubated at 25 ºC and 85% RH during a) 0 hours, b) 24 hours, c) 72 hours or d) 7 days, prior to exposure to artificial rainfall using a rain simulator. Three rain intensities were applied: 60 mm h^{-1}, 100 mm h^{-1} and 150 mm h^{-1}. Values are means of four replicates per treatment. Error bars represent Standard Error.

Discussion

The present results provide a description of the effect of some of the main abiotic factors constraining *C. sake* survival in field conditions, which may directly affect efficacy of treatments. *C. sake* populations increased during the first 72 hours in optimal conditions, in liquid medium and also on the surface of Macabeu grape berries. Populations of *C. sake* without FC increased in sterile PhB, which could be influenced by the reduced trehalose concentration in the original formulation of *C. sake*, whereas multiplication on berries may be also influenced by nutrients present on the fruit surface. When the additive FC was present, *C. sake* grew significantly more in liquid medium, indicating certain nutritive effect of the FC composition, which was also observable on grape berries incubated at 25 ^{0}C and 85% RH, according to results from previous studies on detached table grapes (Calvo-Garrido *et al*., unpublished; PhD thesis Chapter 6). These findings indicate the capacity of *C. sake* to grow without an enriched medium and thereby its ability as a competitive coloniser of fruit surface. *C. sake* also maintained living populations on berry surface after 72 hours of exposure to extreme environmental conditions demonstrating high survival ability. However, when meteorological conditions provided greater constraints for BCA survival (in the second experiment, mean T was 3.7 °C higher and RH was 8.4 % lower than in the first experiment), populations decreased up to 3.5 log units (Fig. 1c). In these adverse environmental conditions for *C. sake* growth, the effect of FC was especially beneficial, since population decrease was around one log unit less when FC was included in the treatment mixture. Thus, the additive FC demonstrated to play an important role during the colonisation process, providing nutrient supply as well as a protective environment for *C. sake* development. Positive effect of the addition of organic natural substances on other BCA´s development and survival has been reported. Several organic and inorganic compounds showed to increase BCA multiplication (Cañamás *et al*., 2008a, Nunes *et al*., 2001, Qin *et al*., 2006, Reeleder, 2004, Tesfagiorgis & Annegarn, 2013). In addition, the combination of BCA's with additives is considered a main strategy to improve biological control of fruit pathogens, even regardless of a BCA survival improvement (Droby *et al*., 2003, Ippolito *et al*., 2005, Lima *et al*., 2005, Tsomlexoglou *et al*., 2002). Consequently, the observed protection of *C. sake* from abiotic factors by FC, added to its efficacy as a standalone product (Calvo-Garrido *et al*., 2013; Calvo-Garrido *et al*. unpublished, PhD thesis Chapter 4) may be regarded as a great advantage of this combinational control strategy. Nonetheless, the protection of *C. sake* was not highly dependent on the FC dose, which improved survival significantly only when conditions were less adverse. Similarly, the higher FC dose (50 g l^{-1}) did not improve *C. sake* survival in controlled conditions (Calvo-Garrido *et al*., unpublished, PhD thesis Chapter 6) or added efficacy when FC was used as a standalone product (Calvo-Garrido *et al*., 2013), compared to the lower dose (25 g l^{-1}). These findings indicate that the lower dose 25 g l^{-1} may represent a more cost effective dose for *C. sake* CPA-1 field applications. The mode of action FC protecting *C. sake* from abiotic factors is still unclear, although may be related to protection from UV radiation, as observed for other additives used with yeast-BCAs (Lahlali *et al*., 2011), whereas it may also protect from fluctuations of T and RH in sub-lethal ranges, since it did not protect *C. sake* from constant limiting conditions of T and RH in climatic chambers (Calvo-Garrido *et al*., unpublished, PhD thesis Chapter 6).

Survival of *C. sake* population was notably affected by abiotic factors during the 24 hours period immediately after application, which showed to be crucial for its establishment. An intense multiplication was observed under optimal growth conditions during the first 24 hours (Fig. 1a), whereas populations were very sensitive to adverse conditions in this period. These trends are in accordance with the results observed in controlled conditions indicating a high sensitiveness of BCA populations in the first period after the application with a remarkable decrease in the first 6-24 hours, while decrease rate was lower after that point (Calvo-Garrido *et al.*, unpublished, PhD thesis Chapter 6). Previous research on *C. sake* (Cañamás *et al.*, 2011) also evidenced rapid population decrease of nearly two log units in the days after field application. However, there are few studies evaluating survival in the period immediately after BCA applications. Tian *et al*. (2004), for example, did not observed such a significant decrease after 24 hours after field application of three selected yeast antagonists on cherries. Nonetheless, rapid population decline after field applications has been also reported for other BCAs, such as *Trichoderma atroviride* in greenhouses (Longa *et al*., 2007), *Pantoea agglomerans* on citrus fruit (Cañamás *et al*., 2008c) or *C. sake* on grapes in previous studies (Cañamás *et al.*, 2011).

A similar trend was observed in the populations of both conditions tested (EXP and COV) in the vineyard experiment, where populations decreased between 0.9 and 1.9 log units during the first 72 hours with a significant decrease in the first 24 hours. In this 24 hours period, differences between the evaluated treatments developed suggesting population sensitivity, while higher reductions in both treatments were observed in the first experiment, when mean temperatures was 5 °C higher during this period. Overall difference in populations between bunches exposed or covered by foliage was about 1 log unit in both replicate experiments. The variations in T and RH may have accounted for differences in population reductions between COV and EXP samples, although sunlight and UV radiation may also be a main differential factor between both conditions.

Differences in populations, such as those in the leaf removal experiment (Fig. 3a, b), may have important practical implications, since one log unit reduction may reduce populations under the concentration threshold for effective suppression. These findings introduce another factor when dealing with BCA field applications, since canopy management through leaf removal may improve BCA spread on bunches (Wurms *et al.*, 2011), while high exposure of treated bunches can compromise BCA survival. Nonetheless, reduced foliage generally improves canopy aeration and reduces *B. cinerea* infection risk (Molitor *et al.*, 2011), indicating that further studies should be carried out to evaluate this interaction and the relative loss in efficacy.

In general, population reductions due to the exposure to outdoor summer conditions were generally higher than those due to simulated rainfall, which reached a maximum of 1.1 log units compared to initial concentration on berries. The *C. sake* population dynamics reductions under the rainfall evidenced two important findings. First, the remarkable reduction during the first part of the rain event (up to 20 mm of rain volume), when most of the population loss was observed in all the conditions tested. A similar pattern has been observed in the study of rainfall effect on the persistence of pesticides on plant tissues. Several copper formulations were washed off up to 50% on grapevine leaves (Molot & Gaimon, 2004) and 80% on apple seedlings (Hunsche *et al*., 2011), after the application of the first 5 mm of rain volume. Hunsche *et al*. (2007) also observed an exponential decrease of mancozeb on apple trees, in accordance to other

studies with the same component (Cabras *et al.*, 2001), whereas herbicide runoff rates were reduced exponentially after the first rain volumes applied (Muller 2004). Moreover, studies with the thrips BCA *Sericothrips staphylinus* reported that the majority of released individuals did not survive after the first 30 mm of simulated rain (Norris *et al.*, 2002). However, the authors found few published works on the population dynamics of BCAs or other microorganisms under rain events and their results were dissimilar. Rain duration did not affect *B. bassiana* conidia retention (Inglis *et al.*, 1995), while significant reduction of *B. thuringiensis* and *P. anomala* efficacy due to simulated rainfall events has been observed (Behle *et al.*, 1997, Jijakli *et al.*, 2002).

The second main finding is the positive effect of an establishment period prior to the exposure to rainfall, which reduced population loss and removed the effect of rain intensity, only observable when there was not an establishment period. Losses due to rainfall of mancozeb were lower when the fungicide was applied 4 to 24 hours before the rain event (Hunsche *et al.*, 2007). Contrarily, a longer drying period prior to rainfall did not reduce copper loss on grapevine leaves (Molot & Gaimon, 2004). In any case, the effect of this period may be different for synthetic or inorganic compounds than for living microorganisms, which have their own multiplication and spread strategies and a specific process of cell-to-surface adhesion. For example, time between application and rain event did not improve efficacy of *B. thuringiensis* (Behle *et al.*, 1997) and rain intensity effect was only significant when *Pseudomonas syringae* pv. *tomato* populations were precariously established on leaves, while populations even increased after a rain event when a 5 days establishment period was provided (Pietrarelli *et al.*, 2006). The effect of rain intensity has been studied by other researchers but results are unclear. It affected irregularly runoff of herbicide applied on maize (Muller *et al.*, 2004) and neither increased the wash off of formulated copper applied on grape leaves (Molot & Gaimon, 2004). However, very low rain intensity (5 mm h^{-1}) provided a linear decrease of mancozeb residues compared to the exponential decrease observed under 5-48 mm h^{-1} intensities. Norris, Memmott *et al*. (2002) observed significant effect of 76-136 mm h^{-1} compared to 13 mm h^{-1}, on *S. staphylinus* survival, while Inglis, Johnson *et al*. (1995) observed a slight effect on the persistence of the *B. bassiana* on alfalfa. In our experiments, the three intensities evaluated, as well as rain volumes, were higher than in most of cited studies and provided a tough test for persistence of *C. sake* plus FC applications, which showed to be elevated. In addition, expected wash off in field conditions might be lower due to protection from direct rain impact provided by vine foliage.

The two main reported findings related to rainfall effect still insist on the sensitiveness of *C. sake* populations soon after the application. Likewise, the occurrence of a establishment period prior to exposure to limiting T and RH conditions reduced up to 1.2 log units the population decrease on table grapes (Calvo-Garrido *et al*., unpublished, PhD thesis Chapter 6). Therefore, a critical period of 0-48 hours has been identified to prevent loss in populations and hence in efficacy and, according to the observed results, recommendations for field applications can be formulated. For example, application should not be carried out if a 24-72 hours period of very high temperatures and low RH is forecasted (Fig. 1c), whereas population loss may be limited if mean daily temperatures stay under 25 °C (Fig. 1b). Dealing with rainfall in field conditions, to avoid a population loss close to 1 log unit, spray applications should be repeated if a cumulative rainfall of 20 mm is recorded in the first 24 hours after application, or 60-120 mm between 24 and 72 hours after the application. Rain events after 72 hours of population

establishment would wash off a significantly lower quantity even after 120 mm cumulative rainfall, which is highly unlikely to happen in Mediterranean-type climate regions. Consequently, it is recommended to apply *C. sake* plus FC 72 hours, or at least 24 hours, before of a forecasted rain event. These practical observations also underline a relative advantage of BCAs compared to inorganic compounds, since treatment persistence may improve due to colonisation ability of microbial antagonists.

In conclusion, *C. sake* demonstrated to survive under unfavourable environmental conditions and persist under intense rain, while this work also defines population dynamics of a yeast BCA under rainfall events for first time. The study evidenced the importance of the first period just after application for *C. sake* survival on grape tissues and also the protective effect of the additive FC. In addition, practical recommendations for *C. sake* plus FC field applications are discussed in order to minimise the effect of abiotic factors and hence achieve reliable control, which may be applicable for field treatments with other yeast BCAs.

Acknowledgements

The authors are grateful to the University of Lleida for Carlos Calvo Garrido PhD grant and the organic production research project and to the European Union for their financial support through the INTERREG REDBIO project. They are also grateful to E. Cases and R. Oró for their technical support and to the L'Olivera winery vineyard managers for their valuable help with field trials and sampling.

References

Abadias M, Usall J, Teixidó N and Viñas I, 2003. Liquid formulation of the postharvest biocontrol agent *Candida sake* CPA-1 in isotonic solutions. *Phytopathology*, 93, 436-442.

An B, Li B, Qin G and Tian S, 2013. Exogenous calcium improves viability of biocontrol yeasts under heat stress by reducing ROS accumulation and oxidative damage of cellular protein. *Current Microbiology*, 65, 122-127.

Andrews Jh, 1992. Biological control in the phyllosphere. *Annual Review of Phytopathology*, 30, 603-635.

Behle Rw, Mcguire Mr and Shasha Bs, 1997. Effects of sunlight and simulated rain on residual activity of *Bacillus thuringiensis* formulations. *Journal of Economic Entomology*, 90, 1560-1566.

Benbow Jm and Sugar D, 1999. Fruit surface colonization and biological control of postharvest diseases of pear by preharvest yeast applications. *Plant Disease*, 83, 839-844.

Cabras P, Angioni A, Garau Vl, *et al.*, 2001. The effect of simulated rain on folpet and mancozeb residues on grapes and on vine leaves. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes*, 36, 609-618.

Calvo-Garrido C, Elmer Pag, Viñas I, Usall J, Bartra E and Teixidó N, 2013. Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1. *Plant Pathology*, 62, 510-519.

Cañamás Tp, Viñas I, Usall J, Casals C, Solsona C and Teixidó N, 2008a. Control of postharvest diseases on citrus fruit by preharvest application of the biocontrol agent *Pantoea agglomerans* CPA-2 part I. Study of different formulation strategies to improve survival

of cells in unfavourable environmental conditions. *Postharvest Biology and Technology*, 49, 86-95.

Cañamás Tp, Viñas I, Usall J, Magan N, Solsona C and Teixidó N, 2008b. Impact of mild heat treatments on induction of thermotolerance in the biocontrol yeast *Candida sake* CPA-1 and viability after spray-drying. *Journal of Applied Microbiology*, 104, 767-775.

Cañamás Tp, Viñas I, Usall J, Torres R, Anguera M and Teixidó N, 2008c. Control of postharvest diseases on citrus fruit by preharvest applications of biocontrol agent *Pantoea agglomerans* CPA-2 part II. Effectiveness of different cell formulations. *Postharvest Biology and Technology*, 49, 96-106.

Cañamás Tp, Viñas I, Torres R, Usall J, Solsona C and Teixidó N, 2011. Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. *Biological Control*, 56, 150-158.

De Clercq D, Cognet S, Pujol M, Lepoivre P and Jijakli Mh, 2003. Development of a SCAR marker and a semi-selective medium for specific quantification of *Pichia anomala* strain K on apple fruit surfaces. *Postharvest Biology and Technology*, 29, 237-247.

Droby S, Wisniewski M, El Ghaouth A and Wilson C, 2003. Influence of food additives on the control of postharvest rots of apple and peach and efficacy of the yeast-based biocontrol product Aspire. *Postharvest Biology and Technology*, 27, 127-135.

Fink U and Volkl W, 1995. The effect of abiotic factors on foraging and oviposition success of the aphid parasitoid, *Aphidius rosae*. *Oecologia*, 103, 371-378.

Glare T, Caradus J, Gelernter W, *et al.*, 2012. Have biopesticides come of age? *Trends in Biotechnology*, 30, 250-258.

Hunsche M, Damerow L, Schmitz-Eiberger M and Noga G, 2007. Mancozeb wash-off from apple seedlings by simulated rainfall as affected by drying time of fungicide deposit and rain characteristics. *Crop Protection*, 26, 768-774.

Hunsche M, Alexeenko A, Damerow L and Noga G, 2011. Rain-induced removal of copper from apple leaves: Influence of rain properties and tank-mix adjuvants on deposit characteristics at the micro scale. *Crop Protection*, 30, 495-501.

Inglis Gd, Johnson Dl and Goettel Ms, 1995. Effects of simulated rain on the persistence of *Beauveria bassiana* conidia on leaves of alfalfa and wheat. *Biocontrol Science and Technology*, 5, 365-369.

Ippolito A, Schena L, Pentimone I and Nigro F, 2005. Control of postharvest rots of sweet cherries by pre- and postharvest applications of *Aureobasidium pullulans* in combination with calcium chloride or sodium bicarbonate. *Postharvest Biology and Technology*, 36, 245-252.

Jijakli Mh, Clercq Dd, Dickburt C and Lepoivre P, 2002. Pre- and post-harvest practical application of Pichia anomala strain K, beta-1,3-glucans and calcium chloride on apples: two years of monitoring and efficacy against post-harvest diseases. *Bulletin OILB/SROP*, 25, 29-32.

Köhl J, Molhoek Wml, Lombaers-Van Der Plas C, Goossen-Van De Geijn H, De Haas L and Gerlagh T, 2002. Effect of environmental factors on conidial germination of the *Botrytis* spp. antagonist *Ulocladium atrum*. *Bulletin OILB/SROP*, 25, 65-68.

Lahlali R, Massart S, Serrhini Mn and Jijakli Mh, 2008. A Box-Behnken design for predicting the combined effects of relative humidity and temperature on antagonistic yeast population density at the surface of apples. *International Journal of Food Microbiology*, 122, 100-108.

Lahlali R and Jijakli Mh, 2009. Enhancement of the biocontrol agent *Candida oleophila* (strain O) survival and control efficiency under extreme conditions of water activity and relative humidity. *Biological Control*, 51, 403-408.

Lahlali R, Raffaele B and Jijakli Mh, 2011. UV protectants for *Candida oleophila* (strain O), a biocontrol agent of postharvest fruit diseases. *Plant Pathology*, 60, 288-295.

Lima G, Curtis Fd, Spina Am and Cicco Vd, 2002. Survival and activity of biocontrol yeasts against powdery mildew of cucurbits in the field. *Bulletin OILB/SROP*, 25, 187-190.

Lima G, Castoria R, Spina Am, De Curtis F and Caputo L, 2005. Improvement of biocontrol yeast activity against postharvest pathogens: Recent experiences. In: Mencarelli F. and Tonutti P., eds. *Proceedings of the 5th International Postharvest Symposium. Acta Horticulturae,* **1-3,** 2035-2040.

Longa C, Elad Y and Pertot I, 2007. Survival of *Trichoderma atroviride* 122F on strawberry phylloplane and in soil. *Bulletin OILB/SROP*, 30, 297-302.

Magan N, 2001. Physiological approaches to improving the ecological fitness of fungal biocontrol agents. In: Butt T. M., Jackson C. and Magan N., eds. *Fungi as biocontrol agents: progress, problems and potential.* Oxon, UK: CABI Publishing, 239-251.

Molitor D, Behr M, Fischer S, Hoffmann L and Evers D, 2011. Timing of cluster-zone leaf removal and its impact on canopy morphology, cluster structure and bunch rot susceptibility of grapes

Journal International des Sciences de la Vigne et du Vin, 45, 149-159.

Molot B and Gaimon C, 2004. Reduction of copper in biological viticulture: study of foliar leaching with simulated rain. *Progres Agricole et Viticole*, 121, 463-468.

Morandi Mab, Mattos Lpv and Santos Er, 2007. Influence of application time on survival, establishment and ability of *Clonostachys rosea* to control *Botrytis cinerea* conidiation on rose debris. *Bulletin OILB/SROP*, 30, 317-320.

Muller K, Trolove M, James Tk and Rahman A, 2004. Herbicide loss in runoff: effects of herbicide properties, slope, and rainfall intensity. *Australian Journal of Soil Research*, 42, 17-27.

Nicot Pc, Decognet V, Fruit L, Bardin M and Trottin Y, 2002. Combined effect of microclimate and dose of application on the efficacy of biocontrol agents for the protection of pruning wounds on tomatoes against *Botrytis cinerea*. *Bulletin OILB/SROP*, 25, 73-76.

Norris Rj, Memmott J and Lovell Dj, 2002. The effect of rainfall on the survivorship and establishment of a biocontrol agent. *Journal of Applied Ecology*, 39, 226-234.

Nunes C, Usall J, Teixidó N, Abadias M and Viñas I, 2001. Improved control of postharvest decay of pears by the combination of *Candida sake* (CPA-1) and ammonium molybdate. *Phytopathology*, 92, 281-287.

Pietrarelli L, Balestra Gm and Varvaro L, 2006. Effects of simulated rain on *Pseudomonas syringae* pv. *tomato* populations on tomato plants. *Journal of Plant Pathology*, 88, 245-251.

Qin Gz, Tian Sp, Xu Y, Chan Zl and Li Bq, 2006. Combination of antagonistic yeasts with two food additives for control of brown rot caused by *Monilinia fructicola* on sweet cherry fruit. *Journal of Applied Microbiology*, 100, 508-515.

Reeleder Rd, 2004. The use of yeasts for biological control of the plant pathogen *Sclerotinia sclerotiorum*. *Biocontrol*, 49, 583-594.

Teixidó N, Viñas I, Usall J, Sanchis V and Magan N, 1998. Ecophysiological responses of the biocontrol yeast *Candida sake* to water, temperature and pH stress. *Journal of Applied Microbiology*, 84, 192-200.

Teixidó N, Torres R, Viñas I, Abadias M and Usall J, 2011. Biological control of postharvest diseases in fruit and vegetables. In: Lacroix C., ed. *Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage* Cambridge, UK: Woodhead Publishing Limited, 364-402. (Food Science, Technology and Nutrition vol. 3.)

Tesfagiorgis Hb and Annegarn Hj, 2013. Evaluation of adjuvants for enhanced management of *Podosphaera xanthii* using potassium silicate and biocontrol agents. *Crop Protection*, 48, 69-75.

Tian Sp, Qin Gz, Xu Y and Wang Ys, 2004. Application of antagonistic yeasts under field conditions and their biocontrol ability against postharvest diseases of sweet cherry. *Acta Botanica Sinica*, 46, 1324-1330.

Tsomlexoglou E, Seddon B and Allan Ej, 2002. Influence of environmental factors on the performance of two biocontrol agents against the grey mould pathogen (*Botrytis cinerea*) in glasshouse-grown tomato crops. *Bulletin OILB/SROP*, 25, 215-218.

Wurms K, Chee A, Elmer P, Agnew R, Wood P and Chee Aa, 2011. Developing new biologically-based products for control of botrytis bunch rot. Part 1: Developing a new natural product for mid-season botrytis control - NP2 moves closer to the market. *Wine and Viticulture Journal*, 26, 64-72.

DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

La presente tesis doctoral evalúa la eficacia de diferentes estrategias alternativas al uso de fungicidas químicos para el control en viña de la podredumbre por *Botrytis cinerea* (PBC), principalmente control biológico con microorganismos antagonistas y la aplicación de productos naturales, con especial énfasis en la efectividad y el modo de acción de la levadura *Candida sake* CPA-1 aplicada con el aditivo Fungicover® (FC).

El control biológico de enfermedades fúngicas como la PBC, especialmente si está basado en la competencia por nutrientes y espacio, requiere dos condiciones básicas: i) la supresión efectiva del patógeno por el antagonista y ii) la presencia del antagonista en altas concentraciones en el momento en que el patógeno se desarrolla (Elad y Stewart, 2004, Elmer y Reglinski, 2006, Jacometti *et al.*, 2010, Teixidó *et al.*, 2011). En nuestro caso de estudio, el potencial de *C. sake* como agente de control biológico (ACB) efectivo contra *B. cinerea*, había sido demostrado en sucesivos estudios *in vitro* e *in vivo* sobre la superficie de fruta de pepita (Teixidó *et al.*, 1998b, Teixidó *et al.*, 1999, Torres *et al.*, 2006, Viñas *et al.*, 1998), así como en aplicaciones de campo en viña (Cañamás *et al.*, 2011). Sin embargo, las condiciones fluctuantes en campo provocan cambios en la relación patógeno-antagonista que pueden afectar la eficacia de los tratamientos. Por lo tanto, para mejorar la efectividad y optimizar la aplicación del ACB se hace necesario un exhaustivo conocimiento de la epidemiología de la enfermedad y de los lugares y los momentos durante la campaña en los que se desarrolla, así como el estudio de la supervivencia de las poblaciones del microorganismo antagonista en campo y de los factores que pueden reducir su presencia en la superficie de la planta.

La epidemiología de *B. cinerea* ha sido escasamente estudiada en zonas de clima mediterráneo y no hay estudios publicados en los que se evalúen las características propias de la PBC en viñedos de la península ibérica. En la presente tesis se estudian varios aspectos de la epidemiología de la enfermedad durante dos campañas en el área de Lleida, con el objetivo de conocer los momentos más importantes para el desarrollo de la enfermedad y la importancia de distintas fuentes de inóculo secundario, para mejorar la efectividad de los tratamientos y conocer mejor su modo de acción. Así mismo, durante los experimentos de aplicación del ACB en campo sobre flores y bayas se realizó un detallado seguimiento de las poblaciones, mientras que complementariamente se realizaron ensayos de campo y laboratorio evaluando la supervivencia bajo diferentes factores abióticos.

Eficacia de los tratamientos con *C. sake* CPA-1 y otras estrategias alternativas a los fungicidas químicos de síntesis

A lo largo de tres campañas vitícolas se realizaron ensayos de campo en los que se aplicaron tratamientos con distintos ACBs y productos naturales (PNs) para observar las reducciones de la incidencia y severidad de la PBC en momento de cosecha. Además, estos experimentos de campo fueron complementados con diferentes ensayos de laboratorio, testando la eficacia de algunos de estos tratamientos en condiciones controladas, con diferentes aislados virulentos de *B. cinerea* y diferentes variedades de uva.

En general, todos los tratamientos evaluados en ensayos de campo y laboratorio con ACBs (*C. sake* CPA-1, *Ulocladium oudemansii*) y/o PNs (quitosán, FC, Protector^{HML}), consiguieron elevadas reducciones de la incidencia y severidad de la infección por *B. cinerea*. Estos resultados confirman el potencial de los productos de origen biológico testados como alternativas efectivas al uso de fungicidas químicos para el control de la PBC, que pueden conseguir niveles aceptables de reducción en diferentes condiciones de campo. Además, las microvinificaciones y la evaluación por un panel de cata entrenado, realizadas durante las campañas 2009 y 2010 utilizando uva de los bloques tratados, no mostraron alteraciones en los principales parámetros de calidad del vino elaborado (Cap.1, Tabla 4).

Los tratamientos con *C. sake* (5×10^7 UFC g^{-1}) junto con el aditivo FC mostraron una alta eficacia en el control de la infección por *B. cinerea* en ensayos controlados en incubación a 21 °C y 100 % HR, condiciones muy favorables para el desarrollo de las cepas virulentas seleccionadas de la colección INRA Bordeaux-Aquitaine (Fig. 1). La reducción observada fue del 75 % de la severidad respecto al control, coincidiendo con resultados previos en ensayos *in vivo* aplicando *C. sake* en la superficie de uva cv. Cabernet Sauvignon (Cañamás *et al*., 2011) y manzanas (Torres *et al*., 2006, Usall *et al*., 2001), mientras que, en condiciones de campo con aplicaciones durante toda la campaña, los niveles de reducción obtenidos fueron similares o superiores.

Los tratamientos de campo en viñedos comerciales que incluyeron aplicaciones durante toda la campaña consistieron en 4-6 aplicaciones con motobomba dependiendo del ensayo, realizadas en estados fenológicos seleccionados entre el inicio de floración hasta siete días antes de cosecha. En cualquier caso, todos incluyeron al menos aplicaciones en floración, pre-cierre de racimo, envero y 21 días antes de cosecha (Cap 1, Tabla 1, 2; Cap. 6, Tabla 1). En el conjunto de los ensayos de campo, la severidad total de PBC en el momento de cosecha fue reducida por estos tratamientos entre el 82 % y el 90 % comparado con el control sin tratar. La incidencia de la enfermedad fue también reducida más del 63 % respecto al control en todos los ensayos.

Estos resultados son especialmente significativos en el caso de las campañas de 2010 en Lleida y 2012 en Burdeos, en las que las condiciones climatológicas fueron muy favorables para el desarrollo de la podredumbre debido a la temperatura moderada, alta HR y abundancia de periodos de humectación foliar después de envero, que tuvieron como consecuencia una alta incidencia y severidad de la PBC en el momento de cosecha (Lleida-2010: 21 %; Burdeos-2012: 12 % y 39 % en cosecha comercial o sobremadurada, respectivamente). Es en estas condiciones altamente favorables al desarrollo del patógeno cuando las estrategias de control biológico suelen aportar un control insuficiente o muy variable (Metz *et al*., 2002). Sin embargo en nuestros estudios la eficacia se mantuvo en niveles muy altos, y se suman a los observados en condiciones de menor presión de enfermedad en 2009 (90 % de reducción de la severidad respecto al control Cap. 1, Tabla 1) y 2006 (83 % de reducción de la severidad respecto al control; Cañamás *et al*. 2011).

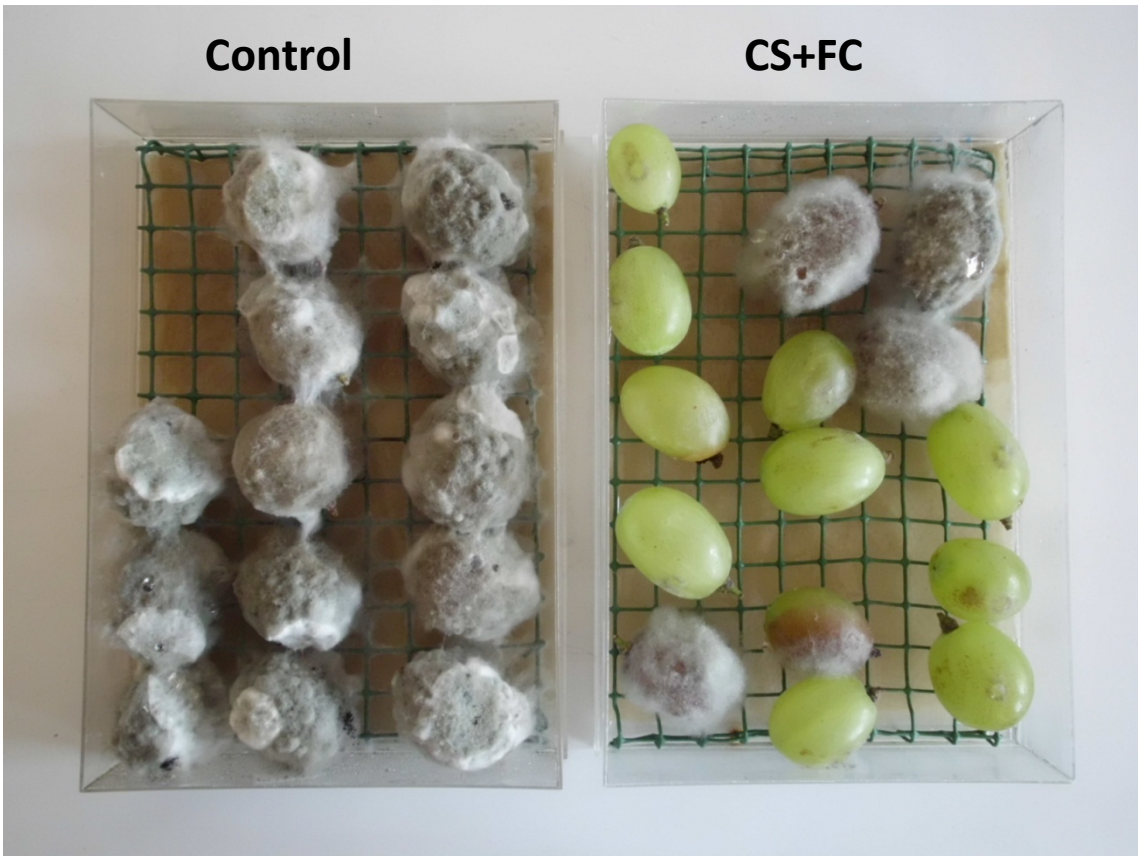


Figura 1 Eficacia del tratamiento *C. sake* y FC contra la infección de *B. cinerea* sobre uvas cv. Italia en condiciones controladas. Las bayas se dejaron sin tratar (Control), o fueron tratadas con la combinación de *C. sake* $5x10^7$ UFC mL^{-1} y FC 50 g L^{-1} (CS+FC). Posteriormente fueron inoculadas con plugs de micelio de tres aislados virulentos de *B. cinerea* (Aislado 213 en la fotografía) y después incubadas a 21 ºC y 100 % HR durante 14 días.

Otros estudios también han demostrado alta eficacia de tratamientos de campo con ACBs contra *B. cinerea* en viña (Meng y Tian, 2009, Reglinski *et al.*, 2005, Reglinski *et al.*, 2010, Wurms *et al.*, 2011, Zahavi *et al.*, 2000), manzana (Droby *et al.*, 2003, Jijakli, 2011, Lima *et al.*, 2003, Scherm *et al.*, 2003, Teixidó *et al.*, 1998b), pera (Benbow y Sugar, 1999), cereza (Ippolito *et al.*, 2005, Tian *et al.*, 2004) o en aplicaciones sobre fresas en invernadero (Guetsky *et al.*, 2002a, Hjeljord *et al.*, 2000, Morandi *et al.*, 2007). Sin embargo, las reducciones mostradas por la combinación de *C. sake* y FC en los estudios realizados, demuestran en general una mayor eficacia y consistencia en el control de *B. cinerea* en campo que la del conjunto de estudios con otros microorganismos y productos naturales analizados en la bibliografía de la presente tesis.

Los ensayos epidemiológicos de aplicación de fungicidas en estados fenológicos seleccionados durante dos campañas (Cap. 5; Fig. 1a, b), destacaron el papel fundamental de los tratamientos en floración para el control efectivo de la PBC, como será discutido en profundidad más adelante. Estos resultados invitaron a explorar la reducción del número de aplicaciones en los tratamientos de *C. sake* con FC. De esta manera, a parte de la aplicación de tratamientos durante toda la campaña, también se evaluó la eficacia de dos aplicaciones focalizadas en floración y cierre de racimo (tratamientos CS+FC x2 y CS+FC25 x2 en Cap. 1; tratamiento "Early season" en Cap. 6). Estos tratamientos consiguieron un control significativo de la incidencia y la severidad de la PBC, aunque las reducciones fueron menores que las

obtenidas con aplicaciones durante toda la campaña. Sin embargo, la diferencia en severidad entre tratamientos consistentes en dos o cuatro aplicaciones en 2010 no fue significativa. Así mismo, en el caso del ensayo realizado en Burdeos, la incidencia y severidad del tratamiento "Early season" en el momento de cosecha comercial fueron similares a las del tratamiento fungicida, por lo que se puede considerar que esta estrategia de dos aplicaciones pre-envero tiene un elevado potencial para controlar la PBC con menor número de aplicaciones. El potencial de esta estrategia ha sido confirmado en posteriores experimentos de campo a mayor escala llevados a cabo por el grupo de patología de la postcosecha del IRTA en la campaña 2011, con equipos de aplicación comerciales y parcelas de 0.5 ha por tratamiento en tres viñedos comerciales de la D.O. Penedés. En estos ensayos se aplicó la combinación *C. sake* y FC a concentraciones menores del ACB y del aditivo comparadas con las empleadas en los ensayos de la presente tesis (*C. sake* $2.5x10^7$ UFC mL^{-1} + FC 50 g L^{-1} y *C. sake* $2.5x10^7$ UFC mL^{-1} + FC 25 g L^{-1}). Se realizaron dos aplicaciones en floración y pre-cierre de racimo, más una aplicación extra en envero en una de las parcelas. Las reducciones obtenidas fueron del 39-85 % de la severidad total respecto al control sin tratar (Calvo-Garrido *et al*., datos no publicados).

Así mismo, las reducciones obtenidas por los tratamientos de *C. sake* y FC durante toda la campaña o focalizados al principio de campaña, fueron similares a las de los tratamientos homólogos aplicando fungicidas, tanto en los ensayos en viñedos de la zona de Lleida (Early, Early+Mid y Early+Late, Cap. 5), como en Burdeos (Fungicide, Cap. 6).

Teniendo en cuenta la efectividad alcanzada, es especialmente remarcable el éxito de la combinación entre *C. sake* CPA-1 y el aditivo basado en ácidos grasos, FC. Este aditivo fue originalmente utilizado para mejorar la dispersión y la persistencia del tratamiento en los tejidos de la planta durante la aplicación, mostrando además un grado de protección sobre las poblaciones de *C. sake* (Cañamás *et al*., 2011). Los resultados de eficacia en cosecha de la campaña de 2009 mostraron además una reducción significativa de la incidencia y severidad de la PBC (50 % y 80 % comparado con el control, respectivamente), que no fue diferente a la alcanzada por la combinación. Sin embargo, cuando las condiciones fueron altamente favorables para el desarrollo de la enfermedad (campaña 2010), el control de la incidencia fue significativamente mayor en los tratamientos con *C. sake* de forma general para una variedad de concentraciones y formas de aplicación. La efectividad de *C. sake* respecto al aditivo concuerda con los resultados de laboratorio (Cap. 6, Fig. 1), en los que la reducción de la severidad de la infección fue un 37 % mayor en el tratamiento combinado CS+FC, que en el de FC solo.

Las aplicaciones con el aditivo FC por si solo contra *B. cinerea* fueron evaluadas en condiciones de campo y laboratorio (Cap. 1, 2, 4, 6), mostrando elevada y consistente eficacia en la protección de tejidos de la viña. En algunos de estos ensayos, se utilizó otro producto comercial (Foodcoat®; Cap. 4), basado en la misma formulación que FC pero a mayor concentración (Fungicover = Foodcoat 68 % + H_2O 32 %).

Los tratamientos con las dosis más altas de las evaluadas de FC (25 y 50 g L^{-1}) redujeron la severidad de la infección entre un 44 y un 96 % en condiciones de laboratorio, dependiendo de la agresividad de los aislados, el cultivar y el método de inoculación (Fig. 2). La efectividad de estas dos concentraciones (25 y 50 g L^{-1}) fue significativamente mayor que la de las dosis menores (5 y 10 g L^{-1}). En los ensayos de campo con FC a 25 y 50 g L^{-1}, la reducción de

incidencia y severidad de la PBC fue de 30-50 % y 74-82 %, respectivamente. El control de la infección de *B. cinerea* por FC evidenció, por tanto, un claro efecto de la dosis del producto, tanto en los ensayos de laboratorio descritos en el Cap. 4 como en la aplicación en campo (Cap. 1, Tabla 3). Sin embargo, entre las dos dosis más efectivas (25 g L^{-1} y 50 g L^{-1}), la reducción de la incidencia en campo fue significativamente diferente pero la severidad no, ni tampoco se detectó un efecto sinérgico entre una mayor dosis de FC y la efectividad de *C. sake*, por lo que la dosis de 25 g L^{-1} representa una manera interesante de reducir el coste del tratamiento sin una pérdida sustancial de efectividad.

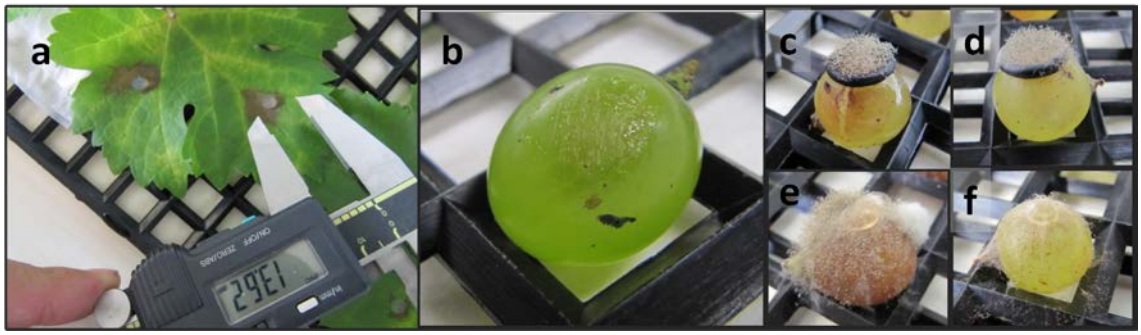


Figura 2 Ensayos de efectividad con el producto basado en ácidos grasos Foodcoat (FC) sobre hojas y bayas cv. Chardonnay. a) Inoculaciones con plugs de micelio sobre hojas de viña; b) imagen de una baya herida mediante la aplicación de papel abrasivo sobre la cutícula. Las figuras c, d, e y f muestran bayas inoculadas con *B. cinerea* tras 7 días de incubación a 22 ºC en cámaras de alta humedad. Las figuras c y e muestran bayas no tratadas, mientras que d y f muestran bayas tratadas con FC 50 g L^{-1}. En uno de los ensayos, las bayas fueron heridas e inoculadas con una suspensión de conidias de *B. cinerea* ($5x10^4$ conidias mL^{-1}; c, d), mientras que en otro ensayo las inoculaciones se realizaron con plugs de micelio (e, f).

En cuanto al modo de acción de FC, los diferentes estudios realizados han permitido definirlo y así comprender mejor la eficacia de FC en campo y las interacciones con *C. sake*. Los estudios realizados durante la estancia en el Ruakura Research Centre, Hamilton, Nueva Zelanda (Cap. 4), evaluaron exhaustivamente la efectividad de la protección de FC usando el producto FoodCoat y comparando con el producto comercial Protector^{HML} (PRT), una formulación de sales de ácidos grasos que se encuentra registrada en Nueva Zelanda para el control de la PBC en viña. Se testaron diferentes concentraciones de ambos productos en pruebas evaluando: germinación, infección de hojas y bayas, en dos variedades (Chardonnay y Sauvignon blanc) y con tres cepas virulentas diferentes de *B. cinerea*, con el objetivo de cuantificar su efectividad y definir su modo de acción en diferentes formas de desarrollo del patógeno.

Los resultados finalmente sugirieron, como se discute ampliamente en el Cap. 4, un modo de acción múltiple incluyendo: 1) Inhibición y alteración del proceso de germinación, 2) bloqueo de infecciones de *B. cinerea* por conidia o micelio mediante establecimiento de una barrera física a través de la formación de una película protectora sobre los tejidos verdes de la viña, 3) protección de heridas en la superficie de las bayas contra la infección, 4) incremento de las poblaciones naturales de levaduras en la superficie de las bayas. A estos modos de acción hay que sumar la elevada supresión de la polilla del racimo por FC, observada en el ensayo de campo en viñas de cv. Merlot en Burdeos (Cap. 6, Fig. 4), ya que el lepidóptero *L. botrana* está

reconocido como un importante vector de transmisión de *B. cinerea* (Fermaud y Giboulot, 1992, Fermaud y Lemenn, 1992). La reducción del número de larvas de *L. botrana* observada en racimos del tratamiento "Full season" (cinco aplicaciones de *C. sake* $5x10^7$ + FC 50 g L^{-1}) fue del 72 % respecto al control, mientras que el tratamiento "Early season" (dos aplicaciones pre-envero de la misma combinación) no redujo la incidencia de larvas. Teniendo en cuenta que las poblaciones de *C. sake* en momento de cosecha fueron similares en ambos tratamientos, el efecto sobre *L. botrana* de las aplicaciones post-envero de FC representa uno de los factores más importantes para explicar la mayor eficacia contra la PBC del tratamiento "Full season" respecto al "Early season" (82 % y 48 % de reducción de severidad comparado con el control, respectivamente). Además de los mecanismos de acción descritos, un último modo de acción para FC sugerido por los resultados es el posible control de la colonización saprofítica de material senescente en el interior de racimos en desarrollo, que será detallada más adelante.

La efectividad mostrada por los productos FC y Protector subraya el interés de los productos naturales basados en ácidos grasos como alternativas efectivas al uso de químicos de síntesis para el control de *B. cinerea*. Otros productos también basados en ácidos grasos han demostrado efectividad contra plagas de insectos (Ma y Gu, 2003, Penaflor *et al.*, 2006) y hongos patógenos (Clausen *et al.*, 2010, Hou, 2008, Karlova *et al.*, 2010, Plockova *et al.*, 1999, Rihakova *et al.*, 2001, Walters *et al.*, 2004, Wang *et al.*, 2002), incluido *B. cinerea* (Bajpai *et al.*, 2009, Cantrell *et al.*, 2008, Hou y Forman, 2000, Montealegre *et al.*, 2010, Puritch *et al.*, 1981). Su eficacia por si solos es en ocasiones limitada y hay pocos trabajos que describan reducciones de enfermedad con aplicaciones de campo (Botta *et al.*, 2009, Palla, 2006). Sin embargo, dadas sus propiedades adicionales como mojantes y su facilidad de extracción y formulación, son buenos candidatos como componentes en tratamientos combinados con otros productos naturales o ACBs.

En el caso de FC, el control obtenido por las aplicaciones en campo de este producto también fue variable en algún caso, a pesar de la eficacia mostrada en la mayoría de ensayos descritos en la presente tesis. Por ejemplo, en los ensayos realizados en viñedos de la popular región vitícola de Marlborough en Nueva Zelanda, los tratamientos consistentes en tres aplicaciones entre envero y cosecha (FC 5, 10 o 25 g L^{-1}) no consiguieron reducciones significativas de la incidencia o la severidad de la PBC respecto al control (datos no publicados). Las condiciones meteorológicas durante la campaña de 2010-11, con un verano relativamente seco poco usual en la zona, propició un escaso desarrollo de la enfermedad y una explosión de la enfermedad leve pero irregular en una situación de elevada madurez. La incidencia y severidad de PBC en los bloques no tratados fue muy baja (20.0 % y 4.2 %, respectivamente), impidiendo la interpretación de los resultados. Sin embargo, la irregularidad de estos resultados evidenció la importancia de las condiciones meteorológicas como factores que determinan un fuerte desarrollo epidémico de *B. cinerea* en momentos concretos. Estos factores pueden hacer inefectivos los tratamientos con productos naturales, como FC. De esta manera, la combinación de ingredientes activos con modos de acción diferentes se considera una estrategia con alto potencial para el control de enfermedades fúngicas como la PBC (Elmer y Reglinski, 2006, Teixidó *et al.*, 2010), capaz de superar la variabilidad de factores implicados en el desarrollo de la enfermedad.

De forma general, los resultados obtenidos muestran las ventajas de la combinación de FC como aditivo para *C. sake*, puesto que es capaz de sumar varios modos de acción contra *B. cinerea* e incrementar la efectividad de los tratamientos de forma directa, pero también favorece la supervivencia y por tanto la eficacia del propio ACB, como será discutido con detenimiento posteriormente.

Sin embargo, tratamientos que incluyen FC tienen la desventaja de un elevado coste del producto, por lo que se hace necesario buscar posibilidades de reducción del coste de los tratamientos durante la campaña. La reducción del número de aplicaciones basada en los resultados de epidemiología mostró ser una estrategia eficaz durante la campaña de 2010. Así mismo, con la reducción de la dosis (25 g L^{-1} respecto a 50 g L^{-1}) se alcanzaron también niveles de reducción elevados. Otra línea de trabajo para reducir coste de los tratamientos puede consistir en la combinación de aplicaciones de FC con otro producto como PRT, con otras cualidades y una menor eficacia, pero coste muy reducido. Protector también ha sido utilizado como aditivo para la aplicación de *U. oudemansii* (http://www.henrymanufacturing.co.nz/products/protectorhml/), mientras que otros productos también han demostrado aumentar la eficacia o supervivencia de tratamientos con ACBs (Droby *et al.*, 2003, Guetsky *et al.*, 2002b, Lima *et al.*, 2005, Nunes *et al.*, 2001, Qin *et al.*, 2006, Reeleder, 2004, Teixidó *et al.*, 2010), algunos de ellos basados en ácidos grasos (McGuire y Hagenmaier, 1996, Tesfagiorgis y Annegarn, 2013). Por lo tanto, dado el potencial de los productos naturales para mejorar la eficacia de los ACBs, futuros estudios deberían continuar evaluando su eficacia y coste para conseguir establecer combinaciones de máxima eficiencia con ACBs y otros PNs.

En los trabajos que componen esta tesis se evaluaron otras estrategias combinadas, además de la aplicación de *C. sake* con el aditivo FC. Durante la campaña de 2009 en Lleida, diferentes combinaciones de *C. sake* y FC, *U. oudemansii* y quitosán fueron evaluadas en campo, aplicando los tratamientos en diferentes momentos del ciclo productivo de la viña en función del modo de acción de los productos (Cap. 1; Tabla 1). Los resultados mostraron reducciones de entre el 73 % y el 82 % de la severidad y el 50 % y el 52 % de la incidencia en los tratamientos combinados. De esta manera, se confirmó la efectividad de *U. oudemansii* y quitosán en condiciones de clima mediterráneo y se añadieron nuevas alternativas para el control de PBC en campo, que *a priori* pueden ofrecer un control menos variable, ya que integran modos de acción complementarios.

Además, los tratamientos combinados que incluían la aplicación de *U. oudemansii* o *C. sake* y FC, consiguieron también reducciones significativas de la podredumbre ácida en momento de cosecha en 2009, cuando se registró alta severidad de esta enfermedad (Cap. 3). En la campaña 2010, con menor severidad en los bloques no tratados, los tratamientos con la combinación de *C. sake* y FC también redujeron significativamente la severidad de esta enfermedad. En ambas campañas, los tratamientos incluyendo *C. sake* presentaron en general menor severidad e incidencia que los tratamientos de FC sin la levadura. Además, tanto 6 aplicaciones (2009) como 4 aplicaciones (2010) de FC solo entre floración y cosecha, no redujeron la severidad de la podredumbre ácida, sugiriendo que el efecto positivo del tratamiento combinado con *C. sake* reside en la aplicación del ACB y no del aditivo.

El control de la podredumbre ácida mostrado por la aplicación de ACBs en esta tesis supone la primera referencia de control biológico de esta enfermedad. Se trata de un resultado importante ya que actualmente no se dispone de tratamientos químicos o biológicos efectivos, tanto en viticultura orgánica como convencional. Así mismo, los tratamientos con estos dos antagonistas (*C. sake* y *U. oudemansii*) fueron capaces de reducir dos importantes enfermedades del viñedo simultáneamente, añadiendo una ventaja, ya que el estrecho espectro de acción es uno de los mayores limitantes para la implementación de tratamientos biológicos (Glare *et al.*, 2012, Montesinos, 2003, Teixidó *et al.*, 2011).

Las interacciones de los tratamientos biológicos contra la PBC con otras enfermedades fúngicas como la podredumbre ácida o con el vector de transmisión *L. botrana*, demuestran la complejidad de los factores implicados en el control de la PBC en campo y la importancia de un enfoque multidisciplinar para el estudio de la efectividad de estos tratamientos, para comprender su modo de acción y mejorar su eficacia.

Epidemiología de *B. cinerea* y eficacia de tratamientos alternativos

Durante las campañas vitícolas de 2009 y 2010, paralelamente a los ensayos de efectividad de productos biológicos, se realizaron ensayos de campo en viñedos de manejo convencional y ecológico para conocer las características propias de la PBC en las condiciones climáticas y varietales de la zona de Lleida. Los resultados obtenidos aportan nueva información epidemiológica sobre esta enfermedad válida para otras regiones vitícolas, especialmente aquellas con climatología de tipo mediterráneo o similar. Los objetivos de estos estudios fueron: 1) identificar los estados fenológicos más importantes para la aplicación de tratamientos y que más contribuyen a la incidencia y severidad de la PBC en momento de cosecha y 2) cuantificar las fuentes de inóculo secundario en el interior del racimo, identificar los estados fenológicos en los que se desarrollan, y evaluar su contribución a la podredumbre en cosecha. Para ello se realizaron cuantificaciones de la incidencia en tejidos necróticos en bloques no tratados, así como experimentos con aplicación de fungicidas en estados fenológicos seleccionados para comprobar su efecto en el posterior desarrollo de *B. cinerea*.

Los resultados del ensayo de aplicación de fungicidas realizados en dos campos de cultivo y durante dos campañas (2009 y 2010; Cap. 5, Fig. 1), mostraron que los tratamientos de floración fueron los más efectivos reduciendo la PBC en ambas campañas. Además, cuando las condiciones meteorológicas fueron favorables para el desarrollo de la PBC, esta eficacia fue incrementada por la adición de otra aplicación de fungicida en pre-cierre de racimo o aplicaciones post-envero, aunque las diferencias en severidad fueron menos evidentes. Estos resultados concuerdan con la alta eficacia de los tratamientos de *C. sake* y FC consistentes en aplicaciones focalizadas en floración y pre-cierre de racimo; (Cap. 1, Tabla 2; CS+FC50 x2, CS+FC25 x2, FC50 x2 y FC25 x2). Aunque estos tratamientos mostraron incidencia significativamente más alta que sus homólogos consistentes en 4 aplicaciones (Cap. 1, Tablas 2 y 3), las diferencias en severidad no fueron significativas y mostraron alta eficacia. Los estados fenológicos en los que se realizaron las aplicaciones (floración y pre-cierre de racimo) se corresponden con los del tratamiento "Early+Mid" del estudio con fungicidas, que fue al más

efectivo en ambas campañas, explicando la elevada eficacia de los tratamientos con *C. sake* y FC focalizados en el principio de la campaña.

La mayor efectividad de los tratamientos fungicidas en floración puso de manifiesto cómo este estado fenológico tiene mayor importancia para el desarrollo de la podredumbre que el periodo post-envero, que es en el que aparecen los síntomas clásicos de la podredumbre en el racimo. Por lo tanto, el desarrollo del ciclo infectivo después de envero depende en gran medida de la infección de las flores y la posterior transformación de estas infecciones en potenciales fuentes de inóculo secundario. Elmer y Michailides (2004) describieron las vías de infección de *B. cinerea* en el viñedo, de las cuales algunas de ellas describen la formación de fuentes de inóculo secundario a partir de ataques de *B. cinerea* en floración, como por ejemplo la formación de infecciones latentes (Vía II) o el desarrollo de inóculo en tejidos necróticos del racimo (Vías III y IV). Otros trabajos también han confirmado la importancia epidemiológica de las infecciones latentes de bayas (Keller *et al*., 2003, Pezet *et al.*, 2003, Sanzani *et al.*, 2012, Viret *et al.*, 2004), o la colonización saprofítica de tejidos florales senescentes (Chebil *et al*., 2003, Elmer *et al.*, 2007, Seyb, 2004, Wolf *et al*., 1997), aunque existen menos estudios que intenten cuantificar su relevancia, mientras que también son escasos los estudios de este tipo en las condiciones del viñedo en clima mediterráneo.

Durante las campañas de 2009 y 2010, se realizaron evaluaciones de tejidos necróticos del interior del racimo de bloques no tratados y también de los bloques tratados con fungicidas en 2010. Las infecciones latentes en bayas aparentemente sanas fueron igualmente evaluadas en 2010, junto con el inóculo superficial sobre las bayas. Para ello, en envero, se tomaron racimos de los que se muestrearon bayas y tejidos necróticos (caliptras, flores abortadas y frutos abortados), que fueron incubados en alta humedad para determinar la incidencia de *B. cinerea* en las muestras (Fig. 3). La contribución de estas potenciales fuentes de inóculo secundario al nivel de podredumbre en momento de cosecha mostró ser variable en función de las condiciones climatológicas que determinan, por un lado, el desarrollo de este inóculo secundario antes de envero y por otro el desarrollo de infecciones en las bayas a partir de estas fuentes de inóculo. Por ejemplo, en el estudio de los bloques no tratados en un campo de agricultura ecológico (Cap. 2, Tabla 2), la alta incidencia de *B. cinerea* en tejidos necróticos en envero no se tradujo en alta incidencia y severidad en cosecha en 2009, cuando el periodo post-envero fue relativamente seco. En 2010, con condiciones favorables para *B. cinerea* después de envero, las infecciones de tejidos necróticos del racimo fueron mucho más reducidas pero la PBC en cosecha muy elevada. En esta misma campaña de 2010, con condiciones meteorológicas post-envero favorables para el desarrollo de la PBC, con los datos de incidencia de *B. cinerea* de las diferentes fuentes potenciales de inóculo secundario en 2010, se realizó un estudio cuantitativo incluido en el Cap. 5. Los resultados mostraron la correlación de las infecciones latentes, infecciones en flores abortadas e infecciones en caliptras senescentes con la incidencia de PBC en cosecha, demostrando la importancia del control de estas fuentes de inóculo cuando las condiciones son favorables a la enfermedad.

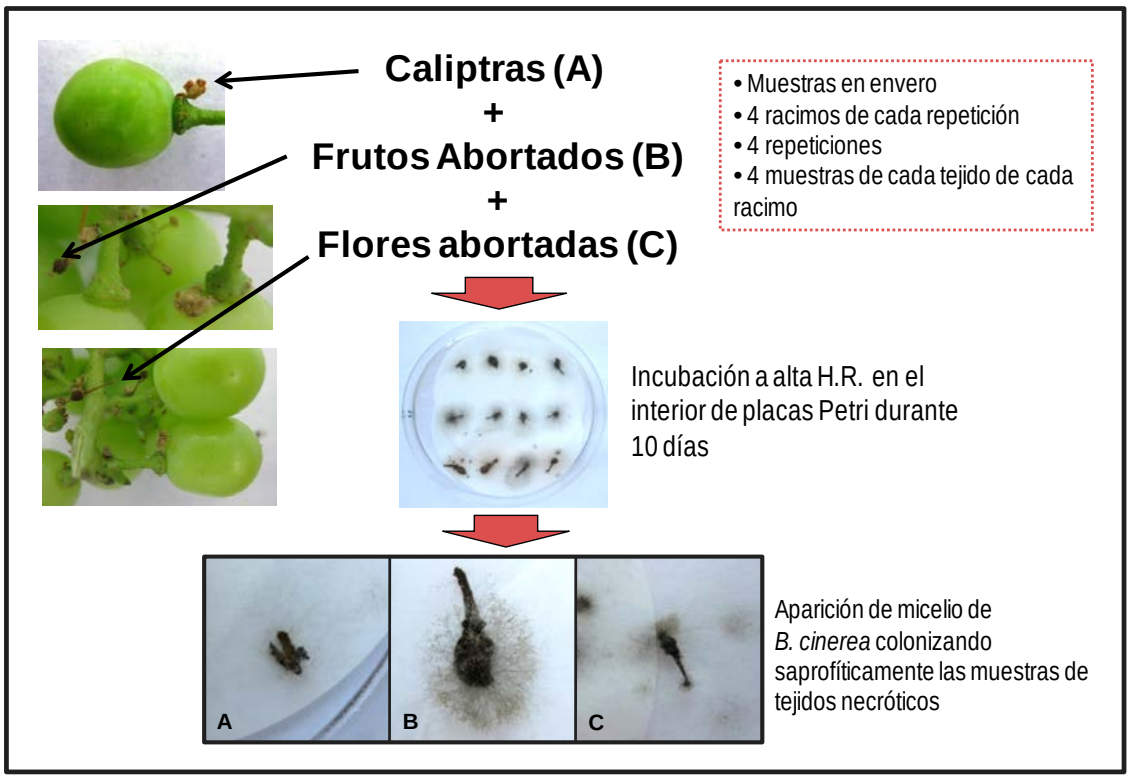


Figura 3 Esquema general del muestreo de tejidos necróticos del interior del racimo, realizado en los ensayos de efectividad y epidemiología en las campañas de 2009 y 2010 en Lleida (Cap. 2, 5). Las muestras se tomaron de bloques no tratados o de bloques tratados con el fungicida iprodiona o con tratamientos biológicos, dependiendo del ensayo. Tras seleccionar las muestras de tejidos necróticos con material estéril, las muestras fueron incubadas para posteriormente evaluar la incidencia de *B. cinerea* con una lupa binocular (Model: SMZ-168, Motic, Barcelona, España; 5x aumentos).

Sin embargo, debido al reducido número de muestras incluido en el estudio cuantitativo, los coeficientes del modelo de regresión obtenido no pueden considerarse como válidos para la predicción de la incidencia, aunque apuntaron a las infecciones latentes como la fuente de inóculo secundario más importante de las evaluadas. En este sentido, sería interesante realizar nuevos estudios sobre este tema para poder corroborar los resultados apuntados.

En el mismo estudio de campo con aplicación de fungicidas (2010), las infecciones latentes de bayas, así como las infecciones en el conjunto de tejidos necróticos evaluados en el estudio en momento de envero, fueron reducidas mayoritariamente por los tratamientos de iprodiona en floración, lo que explicaría parte de la eficacia de los tratamientos en el momento de cosecha. Así mismo, el control de las fuentes de inóculo secundario por los tratamientos alternativos evaluados (Cap. 2) también explicaría parte de la efectividad en cosecha de los mismos tratamientos (Cap. 1), aunque cuantificar la contribución de este efecto es difícil ya que los tratamientos se combinaron con otros tratamientos diferentes después de envero, como se discute más detalladamente en el Cap. 2. En conjunto, los tratamientos alternativos a los químicos, consistentes en dos aplicaciones en floración y una tercera en pre-cierre de racimo, redujeron la incidencia de *B. cinerea* en envero en flores abortadas y caliptras entre el 46 % y el 84 % comparado con el control no tratado. Las infecciones latentes también fueron reducidas por algunos de los tratamientos, aunque los datos mostraron alta variabilidad (datos no publicados). Estos tratamientos consiguieron, por tanto un control efectivo de algunas fuentes

de inóculo secundario del racimo, aunque las tasas de reducción fueron menores que las mostradas por los estudios con fungicidas, en los que el tratamiento "Early+Mid" (tres aplicaciones de iprodiona) redujo el 87.5 % de la incidencia de infecciones latentes y llegó a suprimir completamente la infección y el desarrollo de *B. cinerea* en tejidos necróticos. La iprodiona es una dicarboximida de síntesis con elevada actividad fungitóxica (Rosslenbroich y Stuebler, 2000, USEP, 1998), mientras que en el caso de los tratamientos biológicos se basan en otros modos de acción no directamente tóxicos para el patógeno. Los modos de acción de los tratamientos para el control del inóculo secundario que se proponen, a la vista de los resultados de incidencia en tejidos necróticos (Cap. 2), de los estudios epidemiológicos (Cap. 5) y de efectividad de FC (Cap. 4) son: 1) competencia por nutrientes y supresión de *B. cinerea* en tejidos florales verdes (*C. sake*), 2) actividad antifúngica directa sobre flores y/o tejidos necróticos (FC, quitosán), 3) inducción de resistencia en el huésped (quitosán), 4) competencia en la colonización de tejidos necróticos (*U. oudemansii*) y 5) establecimiento de una barrera física (FC). Todos los tratamientos, con sus diferentes modos de acción fueron efectivos en flores abortadas y caliptras pero no en frutos abortados, mientras que la aplicación de iprodiona no permitió el desarrollo de *B. cinerea* en las muestras evaluadas. Los frutos abortados fueron además el tejido necrótico que presentó mayor incidencia (Cap. 2, Fig. 1), aunque la importancia epidemiológica de estos frutos es relativa ya que su abundancia dentro del racimo es escasa, como se discute en el Cap. 2. La importancia relativa de cada tejido necrótico como fuentes de inóculo puede ser un interesante tema de estudio en el futuro, así como las características y el momento de la infección, dado que los frutos abortados muestreados en estos trabajos presentaron también un marcado crecimiento miceliar y abundante esporulación comparado con los otros tejidos (Fig. 3), haciendo insuficiente el control de los tratamientos biológicos.

Además de los aspectos relacionados con la contribución de los diferentes estados fenológicos y las fuentes de inóculo secundario a la podredumbre en cosecha, los estudios de campo mejoraron el conocimiento de cómo los factores climáticos afectan el desarrollo del patógeno. El número de periodos de lluvia estuvo asociado a un mayor desarrollo de *B. cinerea* en tejidos necróticos en 2009 y fue identificado como un indicador de desarrollo de inóculo secundario, mientras que la humedad foliar fue un factor determinante para la alta incidencia y severidad de PBC observada en 2010 (Cap. 2). Así mismo, las temperaturas superiores a 30 ºC se asociaron a un elevado desarrollo de la podredumbre ácida y menor severidad de PBC en cosecha (Cap. 3), sugiriendo que las variables que miden el tiempo por encima de 30 ºC (Horas T > 30 ºC y Días T_{max} > 30 ºC) son interesantes para estudiar el desarrollo de estas dos enfermedades, como se ha observado en el estudio de otras podredumbres del racimo (Steel y Greer, 2008, Steel *et al.*, 2010, Steel *et al.*, 2011).

Persistencia y supervivencia de *C. sake* CPA-1 en la superficie de la uva

Las poblaciones de *C. sake* tras su aplicación en campo fue evaluada en todos los ensayos de efectividad con este ACB. En ellos, *C. sake* demostró alta capacidad de supervivencia en los experimentos realizados en Lleida y Burdeos. En las condiciones de clima mediterráneo de interior de la D.O. Costers del Segre (Lleida; Cap. 1, Fig. 1 y 2), las poblaciones en flores se redujeron 1.3 log en una semana y las poblaciones en bayas tras envero se redujeron entre

1.3 y 0.2 log en periodos de aproximadamente 2 semanas, comparadas con la concentración inicial justo después de la aplicación. En estas condiciones se hizo necesaria la aplicación repetida de los tratamientos, que elevaron las poblaciones a 5.9-6.3 Log UFC g^{-1} en flores y 4.5-5.2 Log UFC g^{-1} en bayas. En algunos tratamientos con *C. sake* y FC, la última aplicación se realizó en pre-cierre de racimo, aproximadamente dos meses antes del momento de cosecha. En las muestras de estos tratamientos, las poblaciones en bayas se redujeron gradualmente tras la aplicación, pero se mantuvieron por encima de 1.7 Log UFC g^{-1} en todos los muestreos realizados entre envero y cosecha.

El patrón de reducción y recarga de las poblaciones en forma de dientes de sierra, observado en los ensayos de Lleida, no se repitió en las poblaciones analizadas en la región de clima Atlántico de Burdeos (Cap. 6, Fig. 3). En el caso de Burdeos, las poblaciones fueron más altas en flores y las reducciones entre floración y envero fueron menores. En bayas, no decrecieron significativamente entre aplicaciones, y la aplicación sucesiva de *C. sake* no incrementó las poblaciones respecto a las presentes antes del tratamiento, manteniéndose en todo momento por encima de 5 Log UFC g^{-1}. En ambas condiciones climatológicas (Burdeos y Lleida), los tratamientos aplicados mantuvieron una eficacia similar, con las peculiaridades de cada ensayo, como se ha descrito previamente. De forma general y a pesar de las reducciones entre aplicaciones observadas en los ensayos realizados en Lleida, las poblaciones de *C. sake* en campo en las dos condiciones climatológicas y varietales evaluadas fueron similares o superiores a las descritas en otros estudios con aplicaciones de campo de microorganismos antagonistas (Benbow y Sugar, 1999, Ippolito *et al.*, 2005, Karabulut *et al.*, 2003, Lima *et al.*, 2003, Qin *et al.*, 2006, Tian *et al.*, 2004, Zahavi *et al.*, 2000).

Las diferencias observadas en los ensayos de campo, concuerdan con los ensayos de laboratorio que mostraron distinto patrón de supervivencia simulando la climatología atlántica y mediterránea en condiciones constantes con régimen noche/día. En estos ensayos se observó una diferencia de 1 log mantenida en el tiempo entre los tratamientos simulando el clima atlántico (Burdeos; BDX) y el clima mediterráneo (Lleida; LDA) (Cap. 6, Fig. 8). La mayor reducción de las poblaciones de *C. sake* en Lleida también podría estar afectada por la mayor exposición en clima mediterráneo a condiciones limitantes de T y HR, que mostraron su efecto letal sobre la levadura en los experimentos con cámaras climáticas (Cap. 6), o por una mayor exposición al sol y la radiación solar, que fue responsable de la reducción de 1 log en ensayos de campo (Cap. 7, Fig. 3).

El conjunto de ensayos evaluando el efecto de los factores abióticos en la supervivencia de *C. sake* describió la dinámica de las reducciones y demostró la capacidad de este ACB de mantener poblaciones por encima del umbral de detección después de 48-72 horas de exposición a condiciones limitantes para su desarrollo. En los distintos experimentos se cuantificó la pérdida de poblaciones en la superficie de uvas expuestas a alta T (40 °C y 45 °C) en combinación con baja y alta HR (30 % y 100 %) constantes (Cap. 6), así como expuestas a condiciones de verano en clima mediterráneo en ensayos de campo (Cap. 7). La combinación de alta T y alta HR resultó extremadamente letal para *C. sake*, reduciendo drásticamente las poblaciones en 24 horas a niveles bajo el nivel de detección (10^2 UFC g^{-1}) o muy cercanos a él. El efecto negativo de esta combinación sobre los microorganismos concuerda con las observaciones de Cañamás *et al.*, (2008b) evaluando la supervivencia de *Pantoea agglomerans* en

aplicaciones precosecha sobre cítricos y también de otros ACBs (Agra *et al.*, 2012). Este efecto letal de la alta T y HR podría estar relacionado con la mayor conductividad del calor que aportan las propiedades físicas del agua respecto al aire que hacen que el efecto de la alta temperatura sea más directo. Este hecho es el que sugiere que la combinación de alta T y alta humedad es utilizada como tratamiento físico para la prevención de enfermedades postcosecha de fruta (Casals *et al.*, 2010, Elmer *et al.*, 2011, Sisquella *et al.*, 2013).

En cambio, la combinación de alta T y baja HR es más característica de las condiciones limitantes que pueden encontrarse habitualmente en campo, especialmente en zonas de climatología mediterránea. El régimen de 45 °C 30 % mostró elevadas reducciones (4.1 log en 48 horas), que fueron significativamente mayores que a 40 °C y 30 % (2.7 log en 48 horas), seleccionando el segundo como régimen de condiciones limitantes más representativo y de referencia para posteriores ensayos. Las poblaciones en bayas tratadas también decrecieron a 35 °C y 60 % HR en otro ensayo realizado en las mismas cámaras climáticas (datos no publicados). Además, *C. sake* es capaz de crecer a entre 1 °C y 34 °C en medio NYDB, pero sus poblaciones decrecen rápidamente a 37 °C (Usall, 1995). En ensayos *in vitro* sobre medio NYDA, *C. sake* fue capaz de crecer a 30 °C, mientras que la a_ω límite para el crecimiento de *C. sake* en su temperatura óptima (20-25 °C) se definieron entre 0.90-0.92 a_ω (Teixidó *et al.*, 1998a). Por tanto, los resultados sugieren que las altas T, sobre todo por encima de 35 °C representan un factor muy limitante, capaz de reducir las poblaciones de *C. sake* en condiciones de HR tanto limitantes como óptimas para su crecimiento.

La reducción observada después de 72 horas en condiciones limitantes constantes (40 °C y 30 %) fue mayor que las observadas en los ensayos de exposición a condiciones de verano cuando *C. sake* fue aplicada con FC (entre 1.3 y 2.1 log dependiendo del ensayo; Cap 6, Fig. 1b, c), indicando que a pesar del efecto de la radiación solar y las fluctuaciones de las condiciones de T y HR durante el día, las condiciones habituales durante la campaña son menos limitantes que las simuladas en cámaras climáticas. Además, en el viñedo el efecto de los factores abióticos es moderado por otros elementos, como la protección proporcionada por las hojas que se observó en campo (Cap. 7, Fig. 3), en los enyayos comparando las poblaciones en racimos expuestos o cubiertos en el viñedo (Fig. 4a, b). Las condiciones limitantes simuladas representan, por tanto, una referencia de lo que puede ocurrir en campo en momentos concretos, que deben ser controlados ya que pueden reducir la concentración de ACB en la superficie de la uva de forma dramática en poco tiempo.

El efecto del aditivo FC en las poblaciones de *C. sake* fue evaluado en distintos ensayos. La adición de FC en los tratamientos, respecto a la aplicación de *C. sake* sola, no mejoró significativamente la supervivencia de las poblaciones expuestas de forma constante a 40 °C y 30 % HR (Cap. 6, Fig. 6), indicando reducida protección relacionada con altas temperaturas durante periodos largos de tiempo. Sin embargo, cuando las poblaciones en bayas fueron expuestas a factores abióticos fluctuantes (Cap. 7, Fig. 1b, c), los tratamientos con FC en la mezcla mostraron reducciones de aproximadamente 1 log menos en 72 horas. FC también fue beneficioso para el crecimiento de *C. sake* en condiciones óptimas (20-22 °C), tanto en medio líquido como en la superficie de las bayas (Cap. 7, Fig. 1a y 2), aunque las diferencias no fueron significativas en uno de los ensayos (Cap. 6, Fig. 6). Estos resultados indican que la protección ejercida por FC no consistiría en la protección contra situaciones extremas de T y HR, al menos

durante periodos superiores a 6 horas, sino más bien una protección contra las fluctuaciones de estos factores abióticos en rangos sub-letales y contra el efecto de la radiación solar. Cuando las condiciones meteorológicas fueron más moderadas (Cap. 7, Fig. 1b), la dosis más alta de FC (50 g L^{-1}) mostró mejorar la supervivencia de *C. sake* tras 72 horas de exposición, comparado con la dosis baja (25 g L^{-1}). Este efecto no se observó cuando las condiciones fueron más extremas (Cap. 7, Fig. 1c) ni en condiciones limitantes constantes (Cap. 6, Fig. 6) concordando con una hipotética mayor protección de FC en condiciones subletales.

En cuanto a la protección del ACB de las diferentes dosis de FC, en los ensayos de la presente tesis la aplicación de la mayor concentración de FC (50 g L^{-1} comparado a 25 g L^{-1}) no implicó una mejora significativa de la supervivencia de *C. sake* en campo (Cap. 1, Fig. 2). Sin embargo, en un estudio previo con la bacteria *Pantoea agglomerans*, se detectaron diferencias significativas en la persistencia de este ACB cuando fue aplicado sobre cítricos con FC a 20 y 33 g L^{-1}, comparado a 50 g L^{-1} (Cañamás *et al.*, 2008a). Además, como se discute en el apartado de la discusión dedicado a la efectividad, la mejora en efectividad fue escasa, por lo que la dosis de 25 g L^{-1} se considera más apropiada para aplicar como aditivo para *C. sake* en campo.

De forma general, los experimentos revelaron dos interesantes tendencias en cuanto a la reducción de las poblaciones de *C. sake* por causa de factores abióticos a lo largo del tiempo. La primera sería la reducción significativa de las poblaciones durante los primeros momentos tras la aplicación. La pérdida tras 24 horas de exposición varió entre 1 y 3 log, mientras que los ensayos en cámaras climáticas indicaron que la reducción se concentró cuantitativamente en las primeras 6 horas de exposición (1-2.2 log). Esta tendencia se repitió en todos los ensayos realizados, con FC y sin FC, *in planta* o en bayas/clústeres, con o sin la protección del follaje de la viña. La segunda sería el efecto beneficioso de un periodo de establecimiento en condiciones óptimas previo a la exposición a periodos limitantes. Cuando, tras la aplicación de *C. sake* con FC sobre bayas, las muestras fueron incubadas durante 48 horas a 21 °C y 100 % HR, la reducción debida a 48 h de exposición a condiciones limitantes (40 °C, 30 % HR) fue 1.2 log menor que sin periodo de de establecimiento (Cap. 6, Fig. 7).

Estas dos tendencias fueron muy similares a las observadas en los ensayos evaluando las poblaciones de *C. sake* expuestas a periodos de lluvia simulada (Cap. 6, Fig. 4), que representan el primer estudio evaluando el efecto de la lluvia sobre las poblaciones de un ACB fúngico y en el que se utilizó el simulador de lluvia del Departamento de Medio Ambiente y Ciencias del Suelo de la ETSEA, UdL (Fig. 5a, b, c, d). La aplicación de 120 mm de lluvia redujo las poblaciones sobre clústeres de uvas entre 0.4 y 1 log, observándose una fuerte reducción tras la aplicación de los primeros 20 mm de lluvia. Cuando no hubo un tiempo de establecimiento previo a la aplicación de lluvia, la reducción tras 20 mm fue de 0.7 y 1 log, sin una pérdida significativa tras aplicar un mayor volumen de lluvia. Sin embargo, cuando se permitió a las poblaciones establecerse durante 24 horas, 72 horas o 7 días (incubación a 22 °C y 85 % HR), la reducción tras 20 mm fue menor y la aplicación de mayores volúmenes de lluvia redujo gradualmente. Las poblaciones tras 120 mm también se redujeron menos que cuando no hubo establecimiento.

Figura 4 Imágenes del ensayo de campo para evaluar la protección del follaje de la viña sobre las poblaciones de *C. sake* aplicada con FC (Cap. 7; Lleida-2011). Las figuras a) y b) muestran racimos cubiertos (90 % superficie cubierta por hojas) y expuestos (10 % superficie cubierta por hojas) tras un proceso de deshojado manual, respectivamente. Durante 14 días, se monitorizaron las poblaciones de *C. sake* encontrando diferencias significativas entre las poblaciones en racimos con diferente tratamiento de deshojado (Cap. 7, Fig. 3).

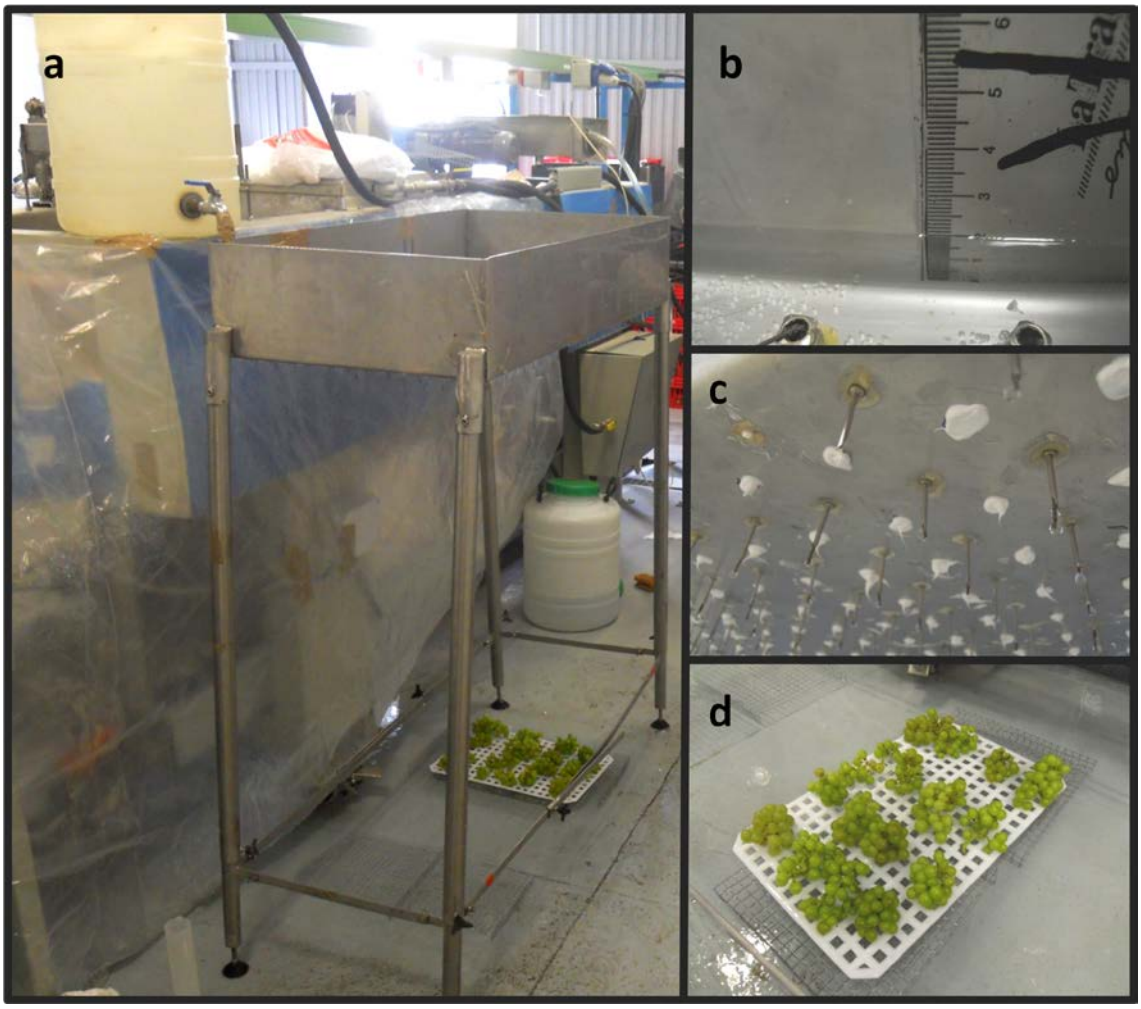


Figura 5 Imágenes del ensayos de persistencia de *C. sake* en la superficie de clústeres de uva cv Macabeu (Cap. 7). a) plano general del simulador de lluvia empleado en los ensayos descritos en el Cap. 7, las muestras se encuentran debajo de la cubeta de goteo; b) detalle del interior de la cubeta y la lámina de agua sobre los goteros, el espesor de la lámina de agua regula la intensidad de la lluvia simulada producida; c) detalle de la parte inferior de la cubeta donde se pueden observar los goteros consistentes en agujas hipodérmicas con un alambre en su interior; d) detalle de los clústeres de aproximadamente 20 uvas expuestos a lluvia simulada.

Así mismo, el efecto de la intensidad de la lluvia aplicada sólo fue significativo cuando no hubo periodo de establecimiento. El efecto positivo de un periodo de establecimiento previo a un episodio de lluvia ha sido descrito en estudios con el fungicida mancozeb (Hunsche *et al.*, 2007) o el patógeno *Pseudomonas syringae* pv. Tomato (Pietrarelli *et al.*, 2006), mientras que no hay otros trabajos evaluando su efecto antes de una exposición a condiciones limitantes de T y HR.

Estas tendencias observadas en la reducción de poblaciones de *C. sake* bajo el efecto de factores abióticos como T, HR, radiación solar y lluvia, mostraron la alta sensibilidad de este ACB en los momentos inmediatamente posteriores a su aplicación, subrayando la importancia de elegir el momento óptimo de aplicación en relación a la meteorología, para reducir la pérdida de ACB en la superficie de los tejidos de la planta.

El conjunto de los estudios discutidos en esta sección cuantificaron y caracterizaron el efecto de factores abióticos influenciando la supervivencia de *C. sake* CPA-1 y, a la vista de la bibliografía consultada, representan uno de los estudios más completos de supervivencia y persistencia de un microorganismo de control biológico en campo y del efecto cuantitativo de los factores abióticos que determinan sus poblaciones.

Aplicaciones prácticas de los resultados obtenidos

Numerosos estudios han evaluado la efectividad de estrategias no químicas para el control de enfermedades fúngicas, incluyendo *B. cinerea* en viña. Sin embargo, en muchos casos la efectividad mostrada en ensayos de laboratorio no se reproduce en ensayos de campo, haciendo reducido el número de productos que llegan a la fase de comercialización (Glare *et al.*, 2012, Köhl *et al.*, 2011, Teixidó *et al.*, 2011). Además, la efectividad de estos productos puede ser variable en función de las condiciones climatológicas particulares de la región, la variedad de uva, o puede ser inefectivo o muy caro si no hay un correcto ajuste de la dosis y el momento de aplicación. Por otra parte, tanto los viticultores de agricultura ecológica como de convencional necesitan nuevos tratamientos, complementarios a las estrategias actualmente utilizadas, que sean efectivos y con un coste reducido, empleando el mínimo de insumos posible.

Por lo tanto, las posibles aplicaciones prácticas para el sector en la aplicación de los tratamientos estudiados en la presente tesis tratamientos biológicos representan gran parte del interés de estos estudios de supervivencia y modo de acción de los tratamientos con ACBs y PNs, así como de epidemiología de las podredumbres del racimo. A continuación se discuten las recomendaciones para la mejora del control de PBC en viña que se extraen de los resultados observados.

Previsión del riesgo de PBC y podredumbre ácida

En los estudios realizados en Lleida durante 2009 y 2010 (Cap. 1, 2, 3 y 5), el desarrollo de *B. cinerea* estuvo asociado a las condiciones climáticas registradas en los campos experimentales evaluados.

La alta incidencia de *B. cinerea* en tejidos necróticos en 2009 coincidió con un mayor periodo de humectación foliar antes de envero (4020 min en 2009 comparado con 1324 min en 2010). A su vez, estos periodos de humectación coincidieron sistemáticamente con episodios de lluvia (ocho en 2009, comparado con cuatro en 2010). Por lo tanto, en las condiciones climáticas de tipo mediterráneo de interior típicas en la zona y en la variedad Macabeu, que presenta racimos muy compactos, el número de episodios de lluvia parece un factor determinante para el desarrollo del inóculo secundario en restos necróticos, que puede considerarse como un indicador sencillo para conocer el posible nivel de desarrollo de estas fuentes de inóculo secundario.

Durante el periodo entre envero y cosecha, el desarrollo de la podredumbre en los racimos también estuvo asociado a las condiciones meteorológicas. La mayor incidencia y severidad de la PBC en 2010 estuvo asociada a mayor precipitación y periodos de humectación foliar más abundantes. Sin embargo, la relación entre los episodios de lluvia y los periodos de humectación foliar no fue tan evidente como la que se observó en el periodo pre-envero.

En cuanto a la podredumbre ácida, la alta incidencia y severidad observadas en los bloques sin tratar en la campaña 2009 estuvo asociada al número de horas en la que la temperatura superó los 30 °C y al número de días en los que la temperatura máxima superó los 30 °C. Estas dos variables que miden el tiempo con T > 30 °C se sugieren como muy interesantes para futuros estudios epidemiológicos de esta enfermedad, y pueden ayudar además a predecir de forma aproximada un mayor desarrollo de la podredumbre ácida a partir del momento de envero.

En cualquier caso, dado que se basan en observaciones realizadas en dos campañas en una zona vitícola, estas recomendaciones deben servir sólo como orientación para prever una mayor o menor presión de las podredumbres del racimo de forma general. Sin embargo, todavía existe una falta de estudios epidemiológicos y sistema de predicción del riesgo sobre estas dos enfermedades, sobre todo en zonas de clima mediterráneo. En otras regiones vitícolas existen sistemas de predicción del riesgo de infección y recomendaciones de aplicación específicas, proporcionados por servicios estatales de extensión e institutos públicos de investigación agronómica, como es el caso de Francia y el modelo desarrollado en el INRA (Fermaud *et al.*, 2003, Fermaud *et al.*, 2010), o Nueva Zelanda con el modelo desarrollado con la colaboración de Plant and Food Research (http://www.botrytis.co.nz/). Mientras tanto, en España se cuenta con algunos sistemas de predicción (Gil *et al.*, 2011, Urbaso, 2003), pero que no siempre están fácilmente disponibles para los viticultores.

Recomendaciones de aplicación

Aplicación de fungicidas de contacto

Los estudios con iprodiona mostraron una alta efectividad de los tratamientos de floración. A la vista de los resultados obtenidos, una estrategia de protección eficiente consistiría en una aplicación durante la floración, complementada con otra aplicación en pre-cierre de racimo si se trata de una parcela con alta severidad de PBC, una región donde las

condiciones son favorables tras el envero, o en general si las condiciones climatológicas son favorables para la enfermedad durante el cierre de racimo; o una aplicación en envero o post-envero si, independientemente de que sea una región o parcela muy sensible a la enfermedad, las condiciones son favorables a la PBC a partir del cierre de racimo. Una sola aplicación en floración sería suficiente para mantener niveles de podredumbre aceptables si las condiciones tras la floración son relativamente secas.

Aplicación de *C. sake* con FC

Los estudios de 2010 en Lleida y de 2012 en Burdeos señalaron el potencial de la estrategia de dos aplicaciones de *C. sake* con FC en la primera parte de la campaña, una en floración y otra en pre-cierre de racimo. Estas dos aplicaciones pueden ser complementadas con otra aplicación en envero o post-envero si las condiciones son altamente favorables (T, HR, percipitación) para la enfermedad en ese periodo, si se detecta un elevado desarrollo de la segunda generación larvaria de *L. botrana* o en caso de un suceso meteorológico adverso como una granizada. Esta estrategia además ha demostrado ser efectiva en estudios de aplicación de *C. sake* y FC a escala comercial en la región vitícola del Penedés (datos no publicados).

Además de las recomendaciones en cuanto al calendario de aplicación de los tratamientos, los resultados de la evaluación de la supervivencia y persistencia de *C. sake* sugirieron una serie de recomendaciones a la hora de aplicar los tratamientos en campo:

La importancia del primer periodo tras la aplicación fue demostrada por la reducción drástica de las poblaciones en este periodo y por el efecto positivo de un periodo de establecimiento, cuando las poblaciones fueron sometidas a episodios de lluvia o a condiciones limitantes de T y HR. Por lo tanto, ya que la reducción de las poblaciones fue muy elevada en las primeras 24 horas tras la aplicación, especialmente durante las 6 primeras horas bajo condiciones limitantes, las aplicaciones de *C. sake* con FC deberían realizarse prioritariamente durante el final de la tarde, cuando las temperaturas se suavizan, manteniéndose en condiciones menos limitantes durante toda la noche. De este modo se minimizan las pérdidas en los momentos iniciales, proporcionando un periodo de establecimiento para las poblaciones en las bayas.

Así mismo, si se pronostican jornadas con temperaturas superiores a 35 °C o episodios de lluvia abundante, es recomendable proveer un tiempo de establecimiento de al menos 48 horas previo a las condiciones limitantes de T y HR o lluvia, ya que el establecimiento de las poblaciones en condiciones favorables para su crecimiento han demostrado mejorar su persistencia y supervivencia a estos factores.

Otra interesante información práctica se refiere a la necesidad de repetir las aplicaciones de campo. Un periodo de 72 horas sin lluvia mejoró significativamente la persistencia a periodos de lluvia posteriores, especialmente en los primeros 20 mm, cuando las poblaciones fueron más sensibles. Por lo tanto, poniendo como límite una pérdida de aproximadamente 0.5-1 log, sería recomendable repetir la aplicación en caso de producirse un episodio de lluvia de más de 20 mm en las primeras 72 horas después de la aplicación.

Efectividad de tratamientos alternativos y combinación de estrategias

Todos los tratamientos de origen biológico evaluados en los diferentes estudios fueron efectivos controlando la PBC en campo en una variedad de uva con alta susceptibilidad a la podredumbre, por lo que su aplicación en viñedos de clima mediterráneo es recomendable y supone un avance para la reducción de tratamientos químicos. Los tratamientos combinados consistentes en la aplicación de una estrategia antes de envero (*C. sake* y FC, *U. oudemansii*, quitosán) y otra a partir de envero (*C. sake* y FC, quitosán) fueron igualmente efectivos que los tratamientos con un solo producto durante toda la campaña. Por lo tanto, estas estrategias de combinación de tratamientos demostraron ser efectivas en las condiciones de viñedo mediterráneo y podrían ser utilizadas en el futuro. La ventaja de las estrategias combinadas sería la de combinar modos de acción diferentes que puedan adaptarse mejor a las variaciones de las condiciones meteorológicas. La eficacia de los tratamientos de *C. sake* y FC, similar o mayor que la de otros productos comerciales, subraya el potencial de *C. sake* para ser comercializado como producto para el control de la PBC en viña.

La combinación de *C. sake* con FC mostró alta efectividad en todos los ensayos y por tanto FC se convierte en un aditivo preferente para la aplicación de esta levadura antagonista. La dosis óptima de aplicación del producto se considera 25 g L^{-1}, ya que no presentó menor efectividad ni redujo la supervivencia de *C. sake* respecto a la dosis de 50 g L^{-1}. La aplicación de dosis menores a 25 g L^{-1} obtuvo menores reducciones de la infección en los estudios realizados en el Cap. 4 y además podría favorecer menos la persistencia de *C. sake*, como se ha observado para *P. agglomerans* aplicada sobre cítricos (Cañamás *et al.*, 2008a).

Sin embargo, el precio del aditivo FC es elevado, y su aplicación a gran escala podría encarecer los tratamientos, por lo que otra estrategia sería combinarlo con otro producto similar (p.e. PRT), con un menor efecto favorable para el desarrollo del ACB y menor efectividad, pero un coste mucho menor. Otro elemento a tener en cuenta en el uso de FC es el cambio del aspecto visual de los racimos tratados, ya que la película de FC sobre la superficie de las bayas adquiere un tono más oscuro y brillante (Fig. 6), que puede no ser deseable en uva de mesa, mientras que en uva de vinificación no es un problema y no afecta la vinificación, como demostraron las microvinificaciones realizadas en 2009 y 2010 (Cap.1, Tabla 5).

Otro factor relacionado con la aplicación de FC es la tendencia observada en 2010 (Cap. 3) que sugiere que los tratamientos post-envero con FC pueden favorecer el desarrollo de la podredumbre ácida, por lo que se deberían limitar las aplicaciones de este producto en años favorables para esta enfermedad.

La combinación de 3 aplicaciones de *U. oudemansii* (BotryZen Ltd) hasta envero y 3 posteriores aplicaciones de *C. sake* con FC fue también muy efectiva. Sin embargo, tras la evaluación de los diferentes modos de acción de estas estrategias, la utilización de *U. oudemansii* podría reducirse a una aplicación en pre-cierre de racimo, para complementar las aplicaciones de floración de *C. sake* con FC añadiendo un modo de acción sobre las fuentes de inóculo secundario en el racimo.

Figura 6 Racimos de uva cv. Merlot tratados con *C. sake* y Fungicover en momento de cosecha. El racimo de la parte izquierda de la fotografía pertenece al control no tratado, mientras que los racimos de la parte derecha pertenecen al tratamiento "Full Season" (Cap. 6; Burdeos, 2012), consistente en cinco aplicaciones de *C. sake* $5x10^7$ UFC mL^{-1} y FC 50 g L^{-1}. En estos últimos se puede observar el aspecto oscuro y brillante producido por la película de Fungicover.

En cuanto a la combinación con técnicas culturales como el deshojado para reducir la humedad y favorecer la ventilación en la viña, los resultados mostraron una pérdida significativa de poblaciones de *C. sake* en los racimos expuestos respecto a los cubiertos por las hojas. Esto implica que se debe valorar la intensidad con la que se realiza esta práctica. Por ejemplo, en regiones con mayor intensidad de la radiación solar, un deshojado más intenso reduciría la supervivencia del ACB y no es recomendable, como también ocurre con el quemado de bayas por el sol debido a un deshojado muy intenso, que puede provocar problemas en la calidad del vino.

En conjunto, los resultados de los ensayos de efectividad en campo demostraron el gran potencial de los tratamientos biológicos utilizados como alternativas al uso de fungicidas químicos de síntesis para el control de la PBC de la viña. Especialmente la combinación de *C. sake* CPA-1 y FC ha confirmado ser una estrategia fiable, con alta eficacia y capaz de reducir la PBC a niveles similares a los de tratamientos fungicidas estándar en una amplia variedad de condiciones. Así mismo, los estudios epidemiológicos y de supervivencia han permitido optimizar las dosis de tratamiento en campo y su momento de aplicación para maximizar su efecto protector. Por tanto, el conjunto de los resultados de la presente tesis doctoral corrobora la efectividad observada en estudios previos y sitúa a *C. sake* CPA-1, aplicada con el aditivo FC, más cerca de su futura aplicación comercial para el control de la PBC, aportando nuevas alternativas para reducir la aplicación de fungicidas químicos de síntesis y prescindir de su uso en un futuro próximo.

REFERENCIAS

Agra O, David DR, Borenshtein M, Shulchany R y Elad Y, 2012. Climate effect on pathogen - biocontrol agents interaction in the tomato - powdery mildew (*Oidium neolycopersici*) pathosystem. *IOBC/WPRS Bulletin*, **78**, 233-237.

Bajpai VK, Kim HR, Hou CT y Kang SC, 2009. Microbial conversion and in vitro and in vivo antifungal assessment of bioconverted docosahexaenoic acid (bDHA) used against agricultural plant pathogenic fungi. *Journal of Industrial Microbiology and Biotechnology*, **36**, 695-704.

Benbow JM y Sugar D, 1999. Fruit surface colonization and biological control of postharvest diseases of pear by preharvest yeast applications. *Plant Disease*, **83**, 839-844.

Botta A, Marín C, Piñol R, Ruz L, Badosa E y Montesinos E, 2009. Study of the mode of action of inicium®, a product developed specifically to overcome transplant stress in strawberry plants. *Acta Horticulturae.* **842**, 721-724.

Cañamás TP, Viñas I, Usall J, Casals C, Solsona C y Teixidó N, 2008a. Control of postharvest diseases on citrus fruit by preharvest application of the biocontrol agent *Pantoea agglomerans* CPA-2 part I. Study of different formulation strategies to improve survival of cells in unfavourable environmental conditions. *Postharvest Biology and Technology*, **49**, 86-95.

Cañamás TP, Viñas I, Usall J, Torres R, Anguera M y Teixidó N, 2008b. Control of postharvest diseases on citrus fruit by preharvest applications of biocontrol agent *Pantoea agglomerans* CPA-2 part II. Effectiveness of different cell formulations. *Postharvest Biology and Technology*, **49**, 96-106.

Cañamás TP, Viñas I, Torres R, Usall J, Solsona C y Teixidó N, 2011. Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. *Biological Control*, **56**, 150-158.

Cantrell CL, Case BP, Mena EE, Kniffin TM, Duke SO y Wedge DE, 2008. Isolation and identification of antifungal fatty acids from the basidiomycete *Gomphus floccosus*. *Journal of Agricultural and Food Chemistry*, **56**, 5062-5068.

Casals C, Teixido N, Vinas I, Llaurado S y Usall J, 2010. Control of *Monilinia* spp. on stone fruit by curing treatments Part I. The effect of temperature, exposure time and relative humidity on curing efficacy. *Postharvest Biology and Technology*, **56**, 19-25.

Chebil S, Roudet J, Dubos B y Ghorbel A, 2003. Table grapes in Tunisia. Optimization of protection with regard to grey mould. *Arboriculture Fruitiere*, **574**, i-v.

Clausen CA, Coleman RD y Yang VW, 2010. Fatty acid-based formulations for wood protection against mold and sapstain. *Forest Products Journal*, **60**, 301-304.

Droby S, Wisniewski M, El Ghaouth A y Wilson C, 2003. Influence of food additives on the control of postharvest rots of apple and peach and efficacy of the yeast-based biocontrol product Aspire. *Postharvest Biology and Technology*, **27**, 127-135.

Elad Y y Stewart A, 2004. Microbial control of *Botrytis* spp. En: Elad Y., Williamson B., Tudzynski P. and Delen N., eds. *Botrytis: biology, pathology and control.* Dordrecht, the Netherlands: Kuwer academic publishers, 223-241.

Elmer PAG y Reglinski T, 2006. Biosuppression of *Botrytis cinerea* in grapes. *Plant Pathology*, **55**, 155-177.

Elmer PAG, Reglinski T, Wurms K y Wood PN, 2007. Enabling technologies: the practical integration of biological and natural products to disease control. In: Butcher M. R., Walker J. T. S. and Zydenbos S. M., eds. *Proceedings of the Future changes in crop protection: Repositioning NZ's Primary Industries. Napier, New Zealand*. 109-116.

Elmer PAG, Spiers TM, Wood P, Reglinski T, Fisher B, Casals C, Calvo-Garrido C y Usall J, 2011. Hot, moist air treatment ("curing") for postharvest suppression of brown rot in

stonefruit caused by *Monilinia fructicola*. En. *Abstract book of the International Congress of Postharvest Pathology. Lleid, Spain.*

Fermaud M y Giboulot A, 1992. Influence of *Lobesia botrana* larvae on field severity of *Botrytis* rot of grape berries. *Plant Disease*, **76**, 404-409.

Fermaud M y Lemenn R, 1992. Transmission of *Botrytis cinerea* to grapes by grape berry moth larvae. *Phytopathology*, **82**, 1393-1398.

Fermaud M, Martinez F, Pieri P, Froidefond G, Sauris P y Delière L, 2003. Pourriture grise de la vigne. Prévision du risque épidémique et protection fongicide. *Phytoma*, **565**, 35-40.

Fermaud M, Roudet J, Sauris P, Druelle L, Blanc G y Lagouarde P, 2010. Implementation of a low-input fungicide strategy in relation to the pattern of grey mold development. *Proceedings of the XV International Botrytis Symposium. Cadiz, Spain.*

Gil E, Llorens J, Landers A, Llop J y Giralt L, 2011. Field validation of DOSAVIÑA, a decision support system to determine the optimal volume rate for pesticide application in vineyards. *European Journal of Agronomy*, **35**, 33-46.

Glare T, Caradus J, Gelernter W, Jackson T, Keyhani N, Köhl J, Marrone P, Morin L y Stewart A, 2012. Have biopesticides come of age? *Trends in Biotechnology*, **30**, 250-258.

Guetsky R, Elad Y, Shtienberg D y Dinoor A, 2002a. Establishment, survival and activity of the biocontrol agents *Pichia guilermondii* and *Bacillus mycoides* applied as a mixture on strawberry plants. *Biocontrol Science and Technology*, **12**, 705-714.

Guetsky R, Shtienberg D, Elad Y, Fischer E y Dinoor A, 2002b. Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology*, **92**, 976-985.

Hjeljord LG, Stensvand A y Tronsmo A, 2000. Effect of temperature and nutrient stress on the capacity of commercial *Trichoderma* products to control *Botrytis cinerea* and *Mucor piriformis* in greenhouse strawberries. *Biological Control*, **19**, 149-160.

Hou CT y Forman RJ, 2000. Growth inhibition of plant pathogenic fungi by hydroxy fatty acids. *Journal of Industrial Microbiology & Biotechnology*, **24**, 275-276.

Hou CT, 2008. New bioactive fatty acids. *Asia Pacific Journal of Clinical Nutrition*, **17**, 192-195.

Hunsche M, Damerow L, Schmitz-Eiberger M y Noga G, 2007. Mancozeb wash-off from apple seedlings by simulated rainfall as affected by drying time of fungicide deposit and rain characteristics. *Crop Protection*, **26**, 768-774.

Ippolito A, Schena L, Pentimone I y Nigro F, 2005. Control of postharvest rots of sweet cherries by pre- and postharvest applications of *Aureobasidium pullulans* in combination with calcium chloride or sodium bicarbonate. *Postharvest Biology and Technology*, **36**, 245-252.

Jacometti MA, Wratten SD y Walter M, 2010. Review: Alternatives to synthetic fungicides for *Botrytis cinerea* management in vineyards. *Australian Journal of Grape and Wine Research*, **16**, 154-172.

Jijakli MH, 2011. *Pichia anomala* in biocontrol for apples: 20 years of fundamental research and practical applications. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, **99**, 93-105.

Karabulut OA, Smilanick JL, Gabler FM, Mansour M y Droby S, 2003. Near-harvest applications of *Metschnikowia fructicola*, ethanol, and sodium bicarbonate to control postharvest diseases of grape in central California. *Plant Disease*, **87**, 1384-1389.

Karlova T, Polakova L, Smidrkal J y Filip V, 2010. Antimicrobial effects of fatty acid fructose esters. *Czech Journal of Food Sciences*, **28**, 146-149.

Keller M, Viret O y Cole FM, 2003. *Botrytis cinerea* infection in grape flowers: Defense reaction, latency, and disease expression. *Phytopathology*, **93**, 316-322.

Köhl J, Postma J, Nicot P, Ruocco M y Blum B, 2011. Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. *Biological Control*, **57**, 1-12.

Lima G, De Curtis F, Castoria R y De Cicco V, 2003. Integrated control of apple postharvest pathogens and survival of biocontrol yeasts in semi-commercial conditions. *European Journal of Plant Pathology*, **109**, 341-349.

Lima G, Castoria R, Spina AM, De Curtis F y Caputo L, 2005. Improvement of biocontrol yeast activity against postharvest pathogens: Recent experiences. En: Mencarelli F. and Tonutti P., eds. *Proceedings of the 5th International Postharvest Symposium. Acta Horticulturae,* **1-3**. 2035-2040.

Ma C y Gu Z, 2003. Insecticidal activity of fatty acid compounds and their potential in controlling pests of cabbage in the field. *Acta Agriculturae Shanghai*, **19**, 101-104.

McGuire RG y Hagenmaier RD, 1996. Shellac coatings for grapefruits that favor biological control of *Penicillium digitatum* by *Candida oleophila*. *Biological Control*, **7**, 100-106.

Meng X y Tian S, 2009. Effects of preharvest application of antagonistic yeast combined with chitosan on decay and quality of harvested table grape fruit. *Journal of the Science of Food and Agriculture*, **89**, 1838-1842.

Metz C, Oerke EC y Dehne HW, 2002. Biological control of grey mould (*Botrytis cinerea*) with the antagonist *Ulocladium atrum*. *Mededelingen Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen Universiteit Gent*, **67**, 353-359.

Montealegre JR, Lopez C, Stadnik MJ, Henriquez JL, Herrera R, Polanco R, Di Piero RM y Perez LM, 2010. Control of grey rot of apple fruits by biologically active natural products. *Tropical Plant Pathology*, **35**, 271-276.

Montesinos E, 2003. Development, registration and commercialization of microbial pesticides for plant protection. *International Microbiology*, **6**, 245-252.

Morandi MAB, Mattos LPV y Santos ER, 2007. Influence of application time on survival, establishment and ability of *Clonostachys rosea* to control *Botrytis cinerea* conidiation on rose debris. *Bulletin OILB/SROP*, **30**, 317-320.

Nunes C, Usall J, Teixidó N, Abadias M y Viñas I, 2001. Improved control of postharvest decay of pears by the combination of *Candida sake* (CPA-1) and ammonium molybdate. *Phytopathology*, **92**, 281-287.

Palla O, 2006. A new formulation based on fatty acids coming from vegetal oils effective against grapevine downy mildew. En: Brunelli A., Canova A. and Collina M., eds. *Proceedings of the Giornate Fitopatologiche 2006. Riccione, Italia*. 17-20.

Penaflor M, Victor SR, Bueno OC, Hebling MJA, Pagnocca FC, Leite AC, Fernandes JB, Vieira PC y da Silva M, 2006. Toxicity of straight-chain fatty acids to leaf-cutting ants *Atta sexdens rubropilosa* (Hymenoptera : Formicidae) and the symbiotic fungus *Leucoagaricus gongylophorus*. *Sociobiology*, **47**, 843-858.

Pezet R, Viret O, Perret C y Tabacchi R, 2003. Latency of *Botrytis cinerea* Pers.: Fr. and biochemical studies during growth and ripening of two grape berry cultivars, respectively susceptible and resistant to grey mould. *Journal of Phytopathology*, **151**, 208-214.

Pietrarelli L, Balestra GM y Varvaro L, 2006. Effects of simulated rain on *Pseudomonas syringae* pv. *tomato* populations on tomato plants. *Journal of Plant Pathology*, **88**, 245-251.

Plockova M, Filip V, Kukackova O, Smidrkal J y Rihakova Z, 1999. Antimicrobial effect of monolauroyglycerol and lauric acid in a model emulsion system. *Czech Journal of Food Sciences*, **17**, 49-54.

Puritch GS, Tan WC y Hopkins JC, 1981. Effect of fatty-acid salts on the growth of *Botrytis cinerea*. *Canadian Journal of Botany-Revue Canadienne De Botanique*, **59**, 491-494.

Qin GZ, Tian SP, Xu Y, Chan ZL y Li BQ, 2006. Combination of antagonistic yeasts with two food additives for control of brown rot caused by *Monilinia fructicola* on sweet cherry fruit. *Journal of Applied Microbiology*, **100**, 508-515.

Reeleder RD, 2004. The use of yeasts for biological control of the plant pathogen *Sclerotinia sclerotiorum*. *Biocontrol*, **49**, 583-594.

Reglinski T, Elmer PAG, Taylor JT, Parry FJ, Marsden R y Wood PN, 2005. Suppression of *Botrytis* bunch rot in Chardonnay grapevines by induction of host resistance and fungal antagonism. *Australasian Plant Pathology*, **34**, 481-488.

Reglinski T, Elmer PAG, Taylor JT, Wood PN y Hoyte SM, 2010. Inhibition of *Botrytis cinerea* growth and suppression of botrytis bunch rot in grapes using chitosan. *Plant Pathology*, **59**, 882-890.

Rihakova Z, Plockova M, Filip V y Smidrkal J, 2001. Antifungal activity of lauric acid derivatives against *Aspergillus niger*. *European Food Research and Technology*, **213**, 488-490.

Rosslenbroich HJ y Stuebler D, 2000. *Botrytis cinerea* - history of chemical control and novel fungicides for its management. *Crop Protection*, **19**, 557-561.

Sanzani SM, Schena L, De Cicco V e Ippolito A, 2012. Early detection of *Botrytis cinerea* latent infections as a tool to improve postharvest quality of table grapes. *Postharvest Biology and Technology*, **68**, 64-71.

Scherm B, Ortu G, Muzzu A, Budroni M, Arras G y Migheli Q, 2003. Biocontrol activity of antagonistic yeasts against *Penicillium expansum* on apple. *Journal of Plant Pathology*, **85**, 205-213.

Seyb A, 2004. *Botrytis cinerea inoculum sources in the vineyard system*. Cristchurch: Lincoln University. PhD Thesis.

Sisquella M, Casals C, Viñas I, Teixidó N y Usall J, 2013. Combination of peracetic acid and hot water treatment to control postharvest brown rot on peaches and nectarines. *Postharvest Biology and Technology*, **83**, 1-8.

Steel CC y Greer DH, 2008. Effect of climate on vine and bunch characteristics: Bunch rot disease susceptibility. En: Adsule P. G., Sawant I. S. and Shikhamany S. D., eds. *Proceedings of the International Symposium on Grape Production and Processing. Acta Horticulturae*, **785**, 253-262.

Steel CC, Greer DH, Samuelian SK y Savocchia S, 2010. Effects of climate and mixed bunch rot infection on *Botrytis cinerea* (grey mould) of grapes: Implications for global climate change and disease management. En. *Abstract book of the XV International Botrytis symposium. Cadiz, Spain*, 57.

Steel CC, Greer LA, Savocchia S y Samuelian SK, 2011. Effect of temperature on *Botrytis cinerea*, *Colletotrichum acutatum* and *Greeneria uvicola* mixed fungal infection of *Vitis vinifera* grape berries. *Vitis*, **50**, 69-71.

Teixidó N, Usall J, Gutierrez O y Viñas I, 1998a. Effect of the antagonist *Candida sake* on apple surface microflora during cold and ambient (shelf life) storage. *European Journal of Plant Pathology*, **104**, 387-398.

Teixidó N, Viñas I, Usall J y Magan N, 1998b. Control of blue mold of apples by preharvest application of *Candida sake* grown in media with different water activity. *Phytopatology*, **88**, 960-964.

Teixidó N, Usall J y Vinas I, 1999. Efficacy of preharvest and postharvest *Candida sake* biocontrol treatments to prevent blue mould on apples during cold storage. *International Journal of Food Microbiology*, **50**, 203-210.

Teixidó N, Usall J, Nunes C, Torres R, Abadias M y Vinas I, 2010. Preharvest strategies to control postharvest diseases in fruits. En: Prusky D. and Gullino M. L., eds. *Postharvest Pathology* (Plant Pathology in the 21st Century; vol. 2). Springer Netherlands, 89-106.

Teixidó N, Torres R, Viñas I, Abadias M y Usall J, 2011. Biological control of postharvest diseases in fruit and vegetables. En: Lacroix C., ed. *Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage* (Food Science, Technology and Nutrition; vol. **3**.). Cambridge, UK: Woodhead Publishing Limited, 364-402.

Tesfagiorgis HB y Annegarn HJ, 2013. Evaluation of adjuvants for enhanced management of *Podosphaera xanthii* using potassium silicate and biocontrol agents. *Crop Protection*, **48**, 69-75.

Tian SP, Qin GZ y Xu Y, 2004. Survival of antagonistic yeasts under field conditions and their biocontrol ability against postharvest diseases of sweet cherry. *Postharvest Biology and Technology*, **33**, 327-331.

Torres R, Teixidó N, Viñas I, Mari M, Casalini L, Giraud M y Usall J, 2006. Efficacy of *Candida sake* CPA-1 formulation in controlling *Penicillium expansum* decay on pome fruit from different mediterranean regions. *Journal of Food Protection*, **69**, 2703-2711.

Urbaso S, 2003. Documento técnico 11/5 - Modelo para el seguimiento del Oidio y la *Botrytis* en la vid. Online (http://www.urbaso.com/dokutek/HToidio_botritis.PDF; Acceso 31-10-2013)

Usall J, 1995. *Control biològic de Penicillium expansum en postcollita de fruita de llavor*. Lleida: Universitat de Lleida, Tesis Doctoral.

Usall J, Teixidó N, Torres R, Ochoa de Eribe X y Viñas I, 2001. Pilot tests of *Candida sake* (CPA-1) applications to control postharvest blue mold on apple fruit. *Postharvest Biology and Technology*, **21**, 147-156.

USEP, 1998. *R.E.D. Facts. Iprodione*. EPA-738-F-98-017. (Prevention, Pesticides and Toxic Substances). Online (http://www.epa.gov/oppsrrd1/REDs/factsheets/2335fact.pdf; Acceso 31-10-2013)

Viñas I, Usall J, Teixido N y Sanchis V, 1998. Biological control of major postharvest pathogens on apple with *Candida sake*. *International Journal of Food Microbiology*, **40**, 9-16.

Viret O, Keller M, Jaudzems VG y Cole FM, 2004. *Botrytis cinerea* infection of grape flowers: light and electron microscopical studies of infection sites. *Phytopathology*, **94**, 850 - 857.

Walters D, Raynor L, Mitchell A, Walker R y Walker K, 2004. Antifungal activities of four fatty acids against plant pathogenic fungi. *Mycopathologia*, **157**, 87-90.

Wang CL, Xing JS, Chin CK y Peters JS, 2002. Fatty acids with certain structural characteristics are potent inhibitors of germination and inducers of cell death of powdery mildew spores. *Physiological and Molecular Plant Pathology*, **61**, 151-161.

Wolf TK, Baudoin A y Martinez-Ochoa N, 1997. Effect of floral debris removal from fruit clusters on botrytis bunch rot of Chardonnay grapes. *Vitis*, **36**, 27-33.

Wurms K, Chee A, Elmer P, Agnew R, Wood P y Chee AA, 2011. Developing new biologically-based products for control of botrytis bunch rot. Part 1: Developing a new natural product for mid-season botrytis control - NP2 moves closer to the market. *Wine and Viticulture Journal*, **26**, 64-72.

Zahavi T, Cohen L, Weiss B, Schena L, Daus A, Kaplunov T, Zutkhi J, Ben-Arie R y Droby S, 2000. Biological control of *Botrytis*, *Aspergillus* and *Rhizopus* rots on table and wine grapes in Israel. *Postharvest Biology and Technology*, **20**, 115-124.

CONCLUSIONES / CONCLUSIONS

Eficacia de los tratamientos con *C. sake* CPA-1 y otras estrategias alternativas a los fungicidas químicos de síntesis

1. Los tratamientos con *C. sake* CPA-1 (5 x 10^7 UFC mL^{-1}) y Fungicover (FC; 50 g L^{-1}) obtuvieron alta eficacia en cuando se aplicaron en campo durante toda la campaña y en experimentos de laboratorio. La reducción de la incidencia y severidad de la PBC fue del 63-67 % y el 82-90 % respecto al control sin tratar, respectivamente. Los niveles de eficacia en campo fueron similares bajo diferentes condiciones climáticas y regiones vitícolas, en viñedos de manejo orgánico y convencional. Además, los tratamientos no alteraron los parámetros de calidad del vino elaborado.

2. Los tratamientos *C. sake* CPA-1 y FC focalizados entre floración y envero también mostraron reducciones significativas de la incidencia y severidad de la PBC (46-51 % y 82-85 %, respectivamente). Esta estrategia de dos aplicaciones focalizadas se sugiere como apropiada para el control de *B. cinerea* en campo.

3. La efectividad del ACB *C. sake* CPA-1 respecto del aditivo FC fue demostrada por los resultados de ensayos de campo y laboratorio. *C. sake* incrementó la reducción de la incidencia y severidad hasta un 37 % y un 28 %, respectivamente, comparado con los tratamientos con FC solo. El modo acción de *C. sake* se confirma como la competencia por espacio y nutrientes en la superficie de los tejidos verdes de la viña (flores y bayas).

4. Todos los tratamientos biológicos evaluados (*C. sake* CPA-1, FC, quitosán y *U. oudemansii*) y las estrategias basadas en su combinación fueron efectivas en el control de la PBC en momento de cosecha, demostrando su potencial como tratamientos alternativos a los fungicidas de síntesis capaces de conseguir alta efectividad de forma fiable.

5. Las aplicaciones de campo con *C. sake* y FC, FC, *U. oudemansii* y quitosán, mostró, de forma general, reducciones significativas de la incidencia de *B. cinerea* en tejidos necróticos del racimo (43-67 % respecto al control), excepto en el caso de los frutos abortados. De esta manera, se añade un modo de acción de estos tratamientos biológicos para reducir la PBC en cosecha, mediante el control de estas fuentes de inóculo secundario.

6. Los dos PNs basados en ácidos grasos evaluados en los diferentes ensayos (FC y PRT), mostraron ser muy efectivos en el control de la infección por *B. cinerea* en hojas y en bayas (44-96 % respecto al control), para un conjunto de aislados del patógeno, dos variedades de viña y diferentes metodologías. La alta eficacia de estos PNs, junto con sus propiedades como mojantes y protectores en la aplicación de ACBs, los sugieren como candidatos para su aplicación en estrategias combinadas con otros PNs y ACBs.

7. La investigación sobre FC y PRT indicó un modo de acción múltiple. El recubrimiento que establece FC sobre la superficie de la uva interfiere con la germinación y la infección por conidias, mientras que es también capaz de bloquear infecciones miceliares y proteger heridas. El modo de acción de PRT mostró ser similar, pero este formulado no alteró la morfología de las conidias germinadas y su protección de las heridas fue ineficaz.

8. La aplicación de tratamientos de campo con FC después de envero mostró: i) incrementar las poblaciones naturales de levaduras residentes en la superficie de racimos Sauvignon blanc, aumentando el número de potenciales antagonistas; ii) reducir significativamente el número de larvas por racimo (cv. Merlot) del vector de PBC *Lobesia botrana*. De este modo, se añaden dos posibles modos de acción para este producto contra *B. cinerea.*

9. La dosis recomendada para la aplicación en campo de FC es de 25 g L^{-1}, ya que cuando fue aplicada una dosis mayor (50 g L^{-1}), no se observó un aumento sustancial de la eficacia ni de la protección proporcionada a las poblaciones de *C. sake*.

10. La severidad de la podredumbre ácida fue reducida por los tratamientos en campo con *C. sake* y Fungicover (2009 y 2010; 40-66 % comparado con el control), así como con *U. oudemansii* (2009; 38-46 % comparado con el control). Estos resultados describen por primera vez el control de esta enfermedad en campo con ACBs y añaden una importante cualidad a estas estrategias para el control de las podredumbres del racimo.

Epidemiología de *B. cinerea* y eficacia de tratamientos alternativos

11. Los estudios con calendarios de aplicación de fungicidas demostraron que, en las condiciones climáticas y varietales evaluadas, el estado fenológico más importante para el desarrollo de la PBC es la floración. Además, la evaluación de fuentes de inóculo secundario de *B. cinerea* señaló que el desarrollo de las infecciones latentes de bayas y la colonización saprofítica de tejidos necróticos se desarrollaron principalmente en este periodo.

12. Los tratamientos de floración con un fungicida de contacto fueron los más efectivos, por lo que se propone una estrategia de control de la PBC consistente en una aplicación en floración, más otra aplicación post envero si las condiciones son favorables a la enfermedad a partir del pre-cierre de racimo. Esta recomendación de aplicación mostró ser eficaz con la aplicación en campo de *C. sake* y FC.

13. Se observó correlación positiva entre la incidencia de la PBC en cosecha y tres fuentes de inóculo secundario (infecciones latentes e infecciones en caliptras y frutos abortados), tratándose del primer estudio que describe un modelo de regresión múltiple con este tipo de variables. Tanto el desarrollo del inóculo secundario como su posterior contribución a la podredumbre en cosecha fueron dependientes de las condiciones meteorológicas.

14. Los frutos abortados fueron el tejido necrótico que presentó mayor incidencia y esporulación de *B. cinerea* en muestras sin tratar (38 % de incidencia comparado con 19 % en flores abortadas y 24 % en caliptras) y además los tratamientos biológicos evaluados no redujeron significativamente su incidencia. Por lo tanto, los frutos abortados suponen una importante fuente de inóculo, aunque su importancia es relativa, ya que su abundancia en el interior del racimo es variable y los tratamientos sí fueron efectivos en otros tejidos necróticos.

15. La incidencia y la severidad de la podredumbre ácida fueron significativamente mayores en condiciones de alta temperatura después del envero. El aumento de la podredumbre ácida estuvo asociado al número de días con temperatura máxima superior a 30 °C, que se sugiere como una variable a tener en cuenta en futuros estudios sobre esta enfermedad.

Persistencia y supervivencia de *C. sake* CPA-1 en la superficie de la uva

16. *C. sake* CPA-1 demostró una alta capacidad de supervivencia en el conjunto de los ensayos de campo, bajo diferentes condiciones climáticas, de variedad de uva y tipo de manejo del viñedo, manteniendo poblaciones entre aplicaciones de 5.5-7 log UFC g^{-1} sobre flores y de 4-6 log UFC g^{-1} sobre bayas, cuando fue aplicada a 5 x 10^7 UFC L^{-1} con FC (50 g L^{-1}).

17. Las poblaciones de *C. sake* sobre bayas en viñedos de clima atlántico (Burdeos) no se redujeron significativamente entre aplicaciones, contrariamente al patrón observado en viñedos de clima mediterráneo (Lleida), con reducciones de aproximadamente 1-2 log entre aplicaciones. Estas diferencias en supervivencia coinciden con los resultados de los ensayos en condiciones simuladas en laboratorio y demuestran la importancia de la climatología para la supervivencia y la efectividad de los ACBs.

18. *C. sake* CPA-1 demostró capacidad de supervivencia ante condiciones limitantes para su desarrollo. Sus poblaciones se redujeron 1-3 log en 24 horas pero mantuvieron niveles detectables incluso tras 72 horas de exposición a condiciones de alta T y baja HR, en ensayos de campo y en condiciones controladas constantes (30 % HR; 40 y 45 °C). La mayor parte de la reducción de la población se produjo durante las primeras 6 horas.

19. El aditivo FC mejoró la supervivencia de las poblaciones de *C. sake* expuestas a condiciones de verano en clima mediterráneo, aunque el efecto protectivo de FC no fue observable bajo condiciones limitantes constantes (30 % HR; 40 °C). El efecto positivo de FC en la supervivencia y eficacia de *C. sake* se ha confirmado, aunque su modo de protección está parcialmente por determinar.

20. La vegetación del viñedo protegió las poblaciones de *C. sake*, ya que las poblaciones fueron aproximadamente 1 log menores en racimos expuestos que en racimos cubiertos por hojas. Los resultados sugieren que las prácticas de deshojado deben planificarse cuidadosamente para no comprometer la supervivencia del ACB.

21. La aplicación de 120 mm de lluvia simulada sobre uvas recién tratadas con *C. sake* y FC redujo las poblaciones de *C. sake* CPA-1 entre 0.7 y 1 log. La principal pérdida se produjo durante la aplicación de los primeros 20 mm.

22. Los resultados evidenciaron la importancia crucial del primer periodo tras la aplicación de *C. sake* para su supervivencia. Las poblaciones de *C. sake* en bayas, expuestas a condiciones limitantes de T y HR, lluvia o radiación solar, mostraron un marcado descenso en el primer periodo después de su aplicación (6-24 horas), mientras que un periodo de establecimiento (24-48 horas; 20-25 °C y 85-100 % HR) incremento significativamente su supervivencia a estos factores abióticos, subrayando la importancia de la elección del momento de aplicación de los tratamientos con ACBs.

23. La información aportada por el conjunto de estudios de supervivencia, en campo y en condiciones controladas, permiten también realizar recomendaciones de aplicación para maximizar la presencia de ACB en la superficie de las bayas, sobre todo en el primer periodo tras la aplicación: i) realizar las aplicaciones *C. sake* con FC prioritariamente por la tarde, ii) proporcionar un tiempo de establecimiento tras la aplicación de *C. sake* (24-48 horas) previo a un periodo con temperaturas superiores a 35 °C o a episodios de lluvia abundante.

Eficàcia dels tractaments amb *C. sake* CPA-1 i altres estratègies alternatives als fungicides químics de síntesi

1. Els tractaments amb *C. sake* CPA-1 (5×10^7 UFC mL^{-1}) i Fungicover (FC; 50 g L^{-1}) van obtenir alta eficàcia quan es van aplicar a camp durant tota la campanya i en experiments de laboratori. La reducció de la incidència i severitat de la PBR va ser del 63-67 % i el 82-90 % respecte al control sense tractar, respectivament. Els nivells d'eficàcia a camp van ser similars sota diferents condicions climàtiques regions vitícoles, en vinyes de maneig ecològic i convencional. A més a mes, els tractaments no van alterar els paràmetres de qualitat del vi elaborat.

2. Els tractaments *C. sake* CPA-1 i FC focalitzats entre floració i verolat també van mostrar reduccions significatives de la incidència i severitat de la PBC (46-51 % i 82-85 %, respectivament). Aquesta estratègia de dues aplicacions focalitzades es suggereix com apropiada per al control de *B. cinerea* a camp.

3. L'efectivitat del ACB *C. sake* CPA-1 respecte de l'additiu FC va ser demostrada pels resultats d'assajos de camp i laboratori. *C. sake* va incrementar la reducció de la severitat i la incidència fins a un 28 % i un 37 %, respectivament, comparat amb els tractament de FC sense l'ACB. El mecanisme d'acció de *C. sake* es confirma com la competència per espai i nutrients a la superfície dels teixits verds de la vinya (flors i baies).

4. Tots els tractaments biològics avaluats (*C. sake* CPA-1, FC, quitosan i *U. oudemansii*) així com les estratègies basades en la seva combinació van ser efectives en el control de la PBC en el moment de collita, demostrant el seu potencial com a tractaments alternatius als fungicides de síntesi capaços d'aconseguir alta efectivitat de manera fiable.

5. Les aplicacions de camp amb *C. sake* i FC, FC, *U. oudemansii* i quitosan, van mostrar, de forma general, reduccions significatives de la incidència de *B. cinerea* en teixits necròtics del interior del carroll (43-67 % respecte al control), excepte en el cas dels fruits avortats. D'aquesta manera, s'afegeix un mecanisme d'acció d'aquests tractaments biològics per reduir la PBC a collita, mitjançant el control d'aquestes fonts d'inòcul secundari

6. Els dos PNs basats en àcids grassos avaluats en els diferents assaigs (FC i PRT), van mostrar ser molt efectius en el control de la infecció per *B. cinerea* en fulles i en baies (44-96 % respecte al control), per a un conjunt d'aïllats del patogen, dues varietats de vinya i diferents metodologies. L'alta eficàcia d'aquests PNs, juntament amb les seves propietats mullants i protectores en l'aplicació d'ACBs, els suggereixen com a candidats per a la seva aplicació en estratègies combinades amb altres PNs i ACBs.

7. La investigació sobre FC i PRT va suggerir un mecanisme d'acció múltiple. El recobriment que estableix FC sobre la superfície del raïm interfereix amb la germinació i la infecció per conidis, mentre que és també capaç de bloquejar infeccions miceliars i protegir ferides. El mecanisme d'acció de PRT va mostrar ser similar, però aquest formulat no va alterar la morfologia dels conidis germinats i la seva protecció de les ferides va ser ineficaç.

8. L'aplicació de tractaments de camp amb FC després de verolat va mostrar: i) incrementar les poblacions naturals de llevats residents a la superfície de raïms Sauvignon blanc, augmentant el nombre de potencials antagonistes; ii) reduir significativament el nombre de larves per carroll (cv. Merlot) del vector de la PBC *Lobesia botrana*. Així, s'afegeixen dos possibles modes d'acció per aquest producte contra *B. cinerea*.

9. La dosi recomanada per a l'aplicació a camp de FC és de 25 g L^{-1}, ja que quan va ser aplicada una dosi major (50 g L^{-1}), no es va observar un augment substancial de l'eficàcia ni de la protecció proporcionada a les poblacions de *C. sake*.

10. La severitat de la podridura àcida va ser reduïda pels tractaments a camp amb *C. sake* i Fungicover (2009 i 2010; 40-66 % comparat amb el control), així com amb *U. oudemansii* (2009; 38-46 % comparat amb el control). Aquests resultats suposen la primera notícia de control d'aquesta malaltia a camp amb ACBs i afegeixen una important qualitat a aquestes estratègies per al control de les podridures del raïm.

Epidemiologia de *B. cinerea* i eficàcia de tractaments alternativos

11. Els estudis amb calendaris d'aplicació de fungicides van demostrar que, en les condicions climàtiques i varietals avaluades, l'estat fenològic més important per al desenvolupament de la PBC és la floració. A més a més, l'avaluació de fonts d'inòcul secundari de *B. cinerea* va assenyalar que el desenvolupament de les infeccions latents de baies i la colonització saprofítica de teixits necròtics es van desenvolupar principalment en aquest període.

12. Els tractaments de floració amb un fungicida de contacte van ser els més efectius, pel que es proposa una estratègia de control de la PBC consistent en una aplicació en floració, més una altra aplicació post-verolat si les condicions són favorables a la malaltia a partir del pre-tancament de carroll. Aquesta recomanació d'aplicació va mostrar ser eficaç amb aplicacions en camp de *C. sake* i FC.

13. Es va observar correlació positiva entre la incidència de la PBC en collita i tres fonts d'inòcul secundari (infeccions latents i infeccions en caliptres i fruits avortats), tractant-se del primer estudi que descriu un model de regressió múltiple amb aquest tipus de variables. Tant el desenvolupament de l'inòcul secundari com la seva posterior contribució a la podridura en collita van ser dependents de les condicions meteorològiques.

14. Els fruits avortats van ser el teixit necròtic que va presentar major incidència i esporulació de *B. cinerea* en mostres sense tractar (38 % comparat amb 19 % en flors avortades i 24 % en caliptres) i a més a més, els tractaments biològics avaluats no van reduir significativament la seva incidència. Per tant, els fruits avortats suposen una important font d'inòcul, encara que la seva importància és relativa, ja que la seva abundància a l'interior del carroll es variable i que els tractaments sí que van ser efectius en altres teixits necròtics.

15. La incidència i la severitat de la podridura àcida van ser significativament majors en condicions d'alta temperatura després del verolat. L'augment de la podridura àcida va estar associat al nombre de dies amb temperatura màxima superior a 30 °C, que es suggereix com una variable a tenir en compte en futurs estudis sobre aquesta malaltia.

Persistència i supervivència de *C. sake* CPA-1 a la superfície del raïm

16. *C. sake* CPA-1 va demostrar una alta capacitat de supervivència en el conjunt dels assaigs de camp, sota diferents condicions climàtiques, de varietat de raïm i tipus de maneig de la vinya, mantenint poblacions entre aplicacions de 5.5-7 log UFC g^{-1} sobre flors i de 4-6 log UFC g^{-1} sobre grans de raïm, quan va ser aplicada a $5x10^7$ UFC L^{-1} amb FC (50 g L^{-1}).

17. La població de *C. sake* a sobre grans de raïm en vinyes de clima atlàntic (Bordeus) no es van reduir significativament entre aplicacions, contràriament al patró observat en vinyes de clima mediterrani (Lleida), amb reduccions d'aproximadament 1-2 log entre aplicacions. Aquestes diferències en supervivència coincideixen amb els resultats dels assaigs en condicions simulades de laboratori i demostren la importància de la climatologia per a la supervivència i l'efectivitat dels ACBs.

18. *C. sake* CPA-1 va demostrar capacitat de supervivència davant condicions limitants per al seu desenvolupament. Les seves poblacions es van reduir 1-3 log en 24 hores però van mantenir nivells detectables fins i tot després de 72 hores d'exposició a condicions d'alta T i baixa HR, en assaigs de camp i en condicions controlades constants (30 % HR, 40 i 45 °C). La principal reducció de la població es va produir durant les primeres 6 hores.

19. L'additiu FC va millorar la supervivència de las poblacions de *C. sake* exposades a condicions d'estiu de clima mediterrani, encara que l'efecte protector de FC no va ser observable sota condicions limitants constants (30 % HR, 40 °C). L'efecte positiu de FC en la supervivència i eficàcia de *C. sake* s'ha confirmat, encara que la seva manera de protecció està parcialment per determinar.

20. La vegetació de la vinya protegeix les poblacions de *C. sake*, ja que les poblacions van ser aproximadament 1 log menors en raïms exposats que en raïms coberts per fulles. Els resultats suggereixen que les pràctiques d'esfullament s'han de planificar amb cura per no comprometre la supervivència de l'ACB.

21. L'aplicació de 120 mm de pluja simulada sobre raïm acabat de tractar amb *C. sake* i FC va reduir les poblacions de *C. sake* CPA-1 entre 0.7 i 1 log. La principal pèrdua es va produir durant l'aplicació dels primers 20 mm.

22. Els resultats van evidenciar la importància crucial del primer període després de l'aplicació de *C. sake* per a la seva supervivència. Les poblacions de *C. sake* sobre grans de raïm, exposades a condicions limitants de T i HR, pluja o radiació solar, van mostrar un marcat descens en el primer període després de la seva aplicació (6-24 hores), mentre que un període d'establiment (24-48 hores; 20-25 °C i 85-100 % HR) va incrementar significativament la supervivència davant aquests factors abiòtics, subratllant la importància de l'elecció del moment d'aplicació dels tractaments amb ACBs.

23. La informació aportada pel conjunt d'estudis de supervivència, a camp i en condicions controlades, permeten també realitzar recomanacions d'aplicació per maximitzar la presència de l'ACB en la superfície dels grans de raïm, sobretot en el primer període després de l'aplicació: i) realitzar les aplicacions de *C. sake* amb FC prioritàriament a la tarda, així com ii) proporcionar un temps d'establiment després de l'aplicació de *C. sake* (24-48 hores), previ a un període amb temperatures superiors a 35 °C o episodis de pluja abundant.

Efficacy of *C. sake* CPA-1 treatments and other alternative strategies to synthetic fungicides

1. The BCA *C. sake* CPA-1 (5 x 10^7 CFU mL^{-1}) plus Fungicover (FC; 50 g L^{-1}) treatments achieved high efficacy in field studies evaluating applications along the whole season and in laboratory studies, reducing *B. cinerea* severity by 82 % to 90 % and field disease incidence by 48 % to 67 %, compared to the untreated controls. The efficacy levels were similar under different climatic conditions in different winegrowing regions, with different vineyard cultural practices and under conventional and organic management. Moreover, treatments did not affect quality parameters of the elaborated wines.

2. The field treatments with *C. sake* CPA-1 and FC applications focused between flowering and veraison also showed significant reductions of BBR incidence and severity (26-51 % and 48-85 %, respectively). Two applications prior to veraison are considered as an appropriate strategy for BBR control with *C. sake* CPA-1 and FC treatments.

3. *C. sake* showed to significantly increase efficacy compared to the additive FC alone in field and laboratory studies. *C. sake* improved incidence and severity control up to 37 % and 28 %, respectively, compared to the treatments with FC alone. Competition for nutrients and space on the surface of grapevine green tissues is considered as the main mode of action of this yeast antagonist.

4. All the biologically-based treatments evaluated (*C. sake* CPA-1, FC, chitosan and *U. oudemansii*), as well as the strategies based on their combinations, significantly reduced *B. cinerea* infection and BBR at harvest. These findings highlight the potential of these treatments as alternative strategies to consistently reduce BBR of grapes.

5. Field treatments with *C. sake* and FC, FC, *U. oudemansii* and chitosan showed, in general, significant reductions of *B. cinerea* incidence on necrotic tissues inside the bunch (43-67 % compared to control), except for the aborted fruits. Therefore, another mode of action, consisting of the suppression of the *B. cinerea* secondary inoculum in necrotic tissues, was evidenced for these treatments.

6. The two fatty acid-based NPs tested (FC and PRT), showed high efficacy controlling severity of *B. cinerea* infection on leaves and berries (44-96 % compared to control), inoculated with selected virulent strains and using two grapevine cultivars. The observed efficacy of these products, as well as their adjuvant and protective effect when applied with BCAs, suggest FC and PRT as valuable candidates for combined strategies with BCAs or other NPs.

7. The investigation on the mode of action of the FC and PRT, suggested a multiple mode of action for these fatty acid based products. The biofilm established by FC applications on grapevine tissues interferes with *B. cinerea* germination and conidial infection, whereas it is able to block mycelial infection and protect berry wounds. PRT mode of action was similar, although it did not altered germ tube morphology and protection of wounds was reduced.

8. The field application of FC after veraison showed: i) an increase of yeast natural populations on grape berries (cv. Sauvignon blanc), thereby increasing the number of potential antagonists; ii) a significant reduction of *L. botrana* larvae in grape bunches (cv. Merlot), reducing potential spread of BBR by this disease vector. This data revealed another two possible modes of action for FC against *B. cinerea*.

9. The recommended dose for field application of FC is 25 g L^{-1}, since the application of a higher dose (50 g L^{-1}) did not substantially improve *B. cinerea* control or *C. sake* survival on grape berries.

10. In the BBR efficacy experiments carried out in Lleida, severity of sour rot was significantly reduced at harvest in plots treated with *U. oudemansii* (2009; 38-46 % compared to control) and *C. sake* plus FC (2009 and 2010; 40-66 % compared to control). This represents the first report of biological control of this disease with microbial antagonists, and represents a qualitative advantage of these BCAs, in order to effectively control grape bunch rots.

Epidemiology of *B. cinerea* and efficacy of biologically-based treatments

11. The fungicide timing experiments indicated that, in the cultivar and climatic characteristics tested the most important phenological stage for BBR development is flowering. In addition, the evaluation of *B. cinerea* secondary inoculum sources at veraison showed that the development of berry latent infections and saprophytic colonisation of necrotic tissues mainly occurred during this period.

12. The treatments including contact fungicide applications at flowering were the most effective ones. Therefore, a strategy consisting of one application at flowering is suggested, with an additional application after veraison if meteorological conditions are favourable to BBR from pre bunch closure onwards. This spray recommendation demonstrated to be applicable for *C. sake* plus FC treatments.

13. Positive correlation was observed between the incidence of BBR at harvest and the incidence of three secondary inoculum sources at veraison (latent infections, infection of necrotic calyptras and infection of aborted fruits). This research is the first describing a multiple regression model relating such variables. However, the development of secondary inoculum was dependent on meteorological conditions before veraison, whereas its later contribution to BBR at harvest also depended on favourable conditions after veraison.

14. The aborted fruits were the necrotic tissues presenting the highest *B. cinerea* incidence in untreated samples (38 % compared to 19 % in aborted flowers and 24 % on calyptras). Moreover, the biologically-based treatments tested did not reduce incidence in these tissues. However, since their frequency in grape bunches is variable, importance of aborted fruits as secondary *B. cinerea* inoculum source may be limited, while treatments were effective controlling incidence on other necrotic tissues.

15. Incidence and severity of sour rot of grapes were significantly higher under meteorological conditions of high temperatures after veraison. The increase of sour rot development was associated to the number of days with maximal temperature over 30 °C, which is suggested as an interesting variable for further investigations on this disease.

Survival and persistence of *C. sake* CPA-1 on grape berry surface

16. Overall, *C. sake* CPA-1 demonstrated high survival ability in the field, under different climatic conditions, grape cultivars and vineyard management, maintaining populations of 5.5-7 log CFU g^{-1} on flowers and 4-6 log CFU g^{-1} on berries, when applied at $5x10^7$ CFU mL^{-1} in combination with FC (50 g L^{-1}).

17. When applied in atlantic climate vineyards (Bordeaux, France), *C. sake* populations on berries did not decrease significantly between applications, contrarily to the findings on field research under mediterranean climate conditions (Lleida, Catalonia, Spain), where reductions of approximately 1-2 log units were observed. These differences were supported by the results of laboratory assays with simulated conditions and demonstrate the importance of climatic conditions on BCA survival and efficacy.

18. *C. sake* CPA-1 showed high survival capacity when exposed to limiting conditions for its development. Its populations were reduced by 1-3 log units in 24 hours, although maintained detectable levels even after 72 hour of exposure to high T and low RH, in the field and under constant 30 % HR and 40-45 °C. The main part of population reduction was observed in first 6 h (1 to 2.2 log units).

19. The additive FC improved *C. sake* survival under Mediterranean summer outdoor conditions, although the FC protective effect on *C. sake* populations was not observable under constant extreme conditions (30 % RH; 40 °C). The FC improvement of survival and efficacy has been confirmed, although the mode of protection remains partially unknown.

20. Vine foliage protected *C. sake* populations on berries, since populations were approximately 1 log unit lower on bunches exposed compared to those covered by vine leaves. These results suggest that leaf removal practices have to be carefully planned to avoid compromising BCA survival.

21. The application of 120 mm of simulated rain on grape berries, immediately after *C. sake* plus FC treatments, reduced the BCA populations between 0.7 and 1 log units. The main population loss was observed after the first 20 mm of rainfall (0.6-0.9 log unit reduction).

22. The results evidenced the crucial importance of the first period just after *C. sake* application for its survival. The *C. sake* populations on berries, exposed to limiting conditions of T and RH, simulated rain or sunlight in the field, showed a remarkable decrease in the first period just after the application (6-24 hours), whereas an establishment time (24-48 hours; 20-25 °C and 85-100 % RH) significantly increased *C. sake* survival under those abiotic factors, highlighting the importance of the application time for BCA treatments.

23. The *C. sake* survival studies, in the field and in controlled conditions, allowed to make spray recommendations to maximise BCA presence on berries, especially in the first period after the application: i) to apply *C. sake* field applications late in the evening and, ii) to provide an establishment time after the application (24-48 hours), prior to a period with temperatures over 35 °C or to a heavy rain event.