Universitat de Girona

DEVELOPMENT OF MOLECULAR MONITORING METHODS AND ASSESSMENT OF THE ENVIRONMENTAL FATE OF THE BIOLOGICAL CONTROL AGENT OF FIRE BLIGHT Pseudomonas Fluorescens EPS62e

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ISBN: 978-84-690-4636-4
Dipòsit legal: GI-286-2007

Doctoral Thesis

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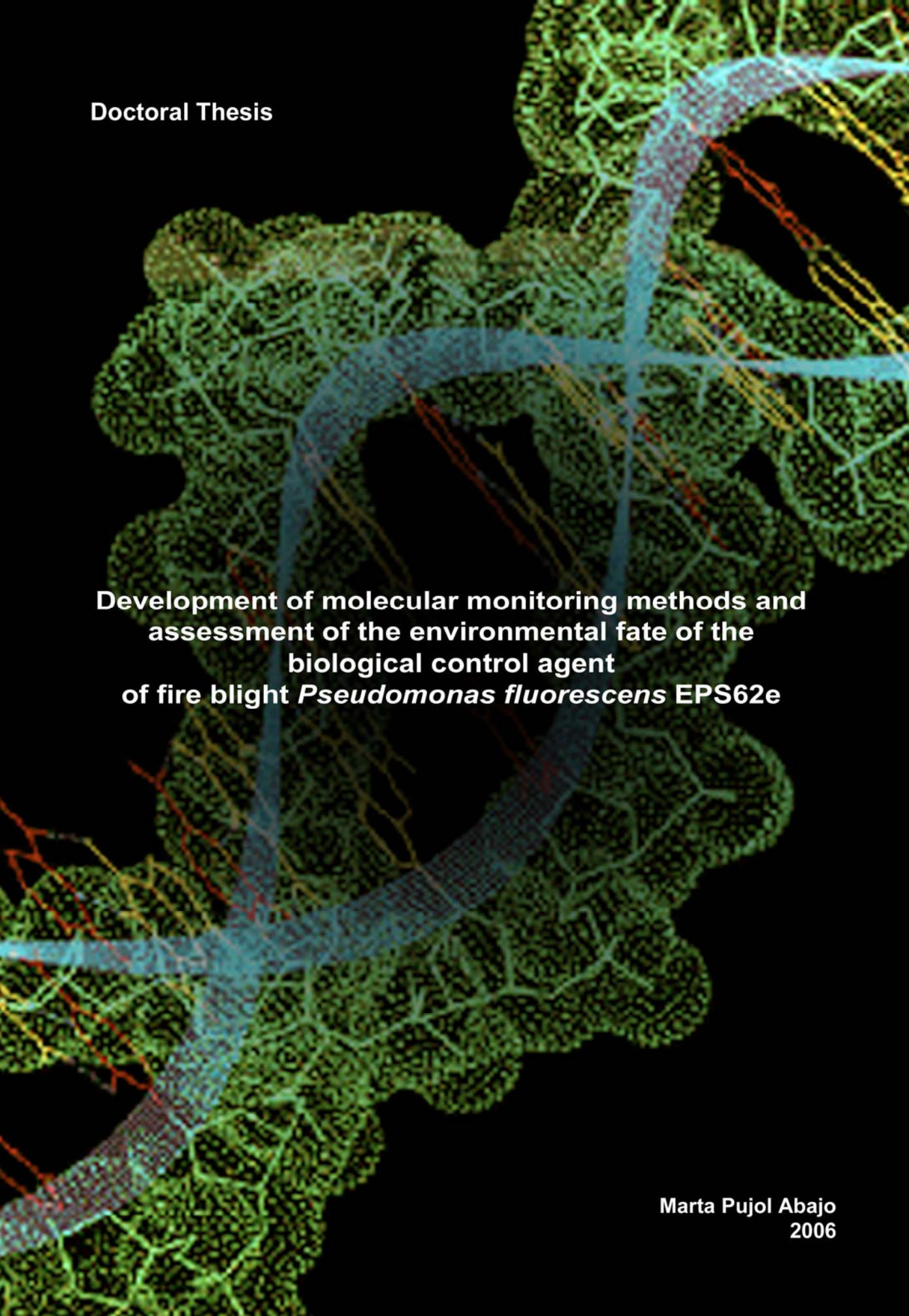

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2006

Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària
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Doctoral Thesis

Development of molecular monitoring methods and assessment of the environmental fate of the biological control agent of fire blight *Pseudomonas fluorescens* EPS62e

Memòria presentada per Marta Pujol Abajo, inscrita al programa de doctorat de Ciències Experimentals i de la Salut, itinerari Biotecnologia, per optar al grau de Doctor europeu per la Universitat de Girona

Marta Pujol Abajo
2006

Emili Montesinos Seguí i **Esther Badosa Romanyó**, catedràtic i professora de l'àrea de Patologia Vegetal del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona,

CERTIFIQUEN

Que la llicenciada en Biologia Marta Pujol Abajo ha dut a terme, sota la seva direcció, el treball amb el títol: "Development of molecular monitoring methods and assessment of the environmental fate of the biological control agent of fire blight *Pseudomonas fluorescens* EPS62e", que presenta en aquesta memòria la qual constitueix la seva Tesi per a optar al Grau de Doctor europeu per la Universitat de Girona.

I per a què consti als efectes oportuns, signen la present a Girona, el 8 de juny de 2006.

Vist-i-plau dels directors de la Tesi

Dr. Emili Montesinos Seguí Dra. Esther Badosa Romanyó

Emili Montesinos Seguí, director dels projectes de recerca "Optimización de los métodos de detección, prevención y control del fuego bacteriano de las rosáceas en España" (Ref. AGL2001-2349-C03-01) i "Evaluación de un procedimiento de bioprotección de fruta y hortalizas frescas para el control de podredumbres fúngicas y de patógenos causantes de toxiinfecciones alimentarias" (Ref. CAL03-084)

Marta Pujol Abajo, que inscriu la tesi doctoral titulada "Development of molecular monitoring methods and assessment of the environmental fate of the biological control agent of fire blight *Pseudomonas fluorescens* EPS62e" amb el número de registre 704 de la UdG (25 d'octubre del 2004)

DECLAREN

Que aquesta tesi s'ha realitzat en el marc dels esmentats projectes i està sotmesa a la propietat intel·lectual compartida amb els investigadors del grup de recerca de Patologia Vegetal de la Universitat de Girona que participen en aquests projectes (Article 2. apartat 2, RD 1326/2003 del 24-10-2003; Llei de la Propietat Intel·lectual, RD 1/1996 del 12-04-1996).

I per a què consti als efectes oportuns, signen la present a Girona el 8 de juny de 2006.

Vist-i-plau

Dr. Emili Montesinos Seguí Marta Pujol Abajo

This work has been performed within the framework of the research projects "Optimización de los métodos de detección, prevención y control del fuego bacteriano de las rosáceas en España" (Ref. AGL2001-2349-C03-01) funded by the *Comisión Interministerial de Ciencia y Tecnología*, Spain; and "Evaluación de un procedimiento de bioprotección de fruta y hortalizas frescas para el control de podredumbres fúngicas y de patógenos causantes de toxiinfecciones alimentarias" (Ref. CAL03-084) funded by the *Ministerio de Ciencia y Tecnología* and the *Instituto Nacional de Investigación Agropecuaria*, Spain; and with the research grant FP-2001-2130 of the *Ministerio de Educación y Ciencia*, Spain. Part of the work described in this thesis was performed during a research stay in the *Institut National de la Recherche Agronomique* (INRA), Angers, France.

SUMMARY

Erwinia amylovora is a plant pathogenic bacterium that causes economically important losses to pear and apple production around the world. Traditionally, fire blight management has been based on chemical control strategies, mainly based in cooper compounds and certain antibiotics depending on the legislation of each country. However, the reiterative use of chemical pesticides has carried negative effects such as the appearance of pathogen resistant strains and the presence of chemical residues in food and in the environment. To overcome these drawbacks, other strategies have been developed, such as biological control. *Pseudomonas fluorescens* EPS62e was selected in the Plant Pathology Group of the University of Girona and is being developed as a biological control agent of fire blight because of its high efficacy controlling *E. amylovora* infections in immature pear fruits, flowers and potted pear plants.

In the present work, we aimed to contribute to the development of the biocontrol agent *P. fluorescens* EPS62e by means of the design of a molecular monitoring method based on Real-time PCR, and the assessment of colonisation and survival of EPS62e in apple and pear after field release.

The development of a Real-time PCR methodology involved (i) the detection of polymorphisms within EPS62e genome, (ii) the selection of differential amplified fragments which discriminate EPS62e from other strains to design sequence characterised amplified regions (SCAR) as molecular markers, (iii) the evaluation of the specificity of the PCR based on SCAR markers, and (iv) the design of a Real-time PCR in an internal region of the SCAR sequences. Two PCR-based methods that did not require previous knowledge of the target strain genome, random amplified polymorphic DNA (RAPD) and Unspecific-PCR (U-PCR), were used to find natural polymorphisms within EPS62e genome. Both methods brought out fingerprinting profiles that allowed the discrimination of EPS62e from other strains of the same species. Three differential amplified fragments from RAPD and U-PCR profiles were sequence characterised as SCAR markers. A primer pair was designed for each SCAR marker, and the specificity was evaluated in PCR with 161 strains belonging to the same species, 75 strains of closely related species, and 61 field samples. Two SCAR markers were fully specific for the detection of EPS62e, while the third SCAR marker designed gave unspecific amplification with other *P. fluorescens* strains. On the bases of the specific SCAR markers, two Real-time PCR designs were developed with TaqMan® probes. Both designs of Real-time PCR were specific for EPS62e, with the detection level situated at 10^2 CFU g fw^{-1} and the standard curve linear over a five-log range.

During the development and optimisation of Real-time PCR, two classical monitoring methods, based on CFU-counting and most probable number (MPN) determination on selective culture media, were coupled to SCAR markers and compared for monitoring the cultivable population of EPS62e on pear leaf surfaces under controlled environment conditions. Both methods were useful to track EPS62e since no significant differences were observed between them. The EPS62e cultivable population level showed a progressive decrease from 10^7 to 10^5 – 10^4 CFU g fw^{-1} during the first 17 days, and then it remained stable for 11 days until the end of the assay. Even though both techniques were useful, CFU-counting on selective media was selected for further assays.

To validate Real-time PCR for the monitoring of EPS62e, it was used simultaneously with CFU-counting method in several trials on apple flowers and leaves, and the results were statistically compared and contrasted. Trials were performed under

greenhouse and field conditions in Maine-et-Loire region (France) during springtime 2004. A good correlation of population levels estimated by Real-time PCR and CFU-counting methods was found in apple blossom trials. The biocontrol agent EPS62e showed to be an efficient coloniser of apple flowers under greenhouse and field conditions, reaching high population levels from 10^7 to 10^8 CFU/blossoms. This population was maintained even during fruit formation and growth, 54 days after treatment, and up to 99% of population was restricted to the calyx area of the fruit. A different situation was observed in apple leaf trials, where population levels of EPS62e decreased with time. In apple leaves, the population levels estimated by Real-time PCR and CFU-counting methods significantly differed. In general, Real-time PCR values were higher than cultivable values. It was concluded that two factors were mainly involved in the difference observed: the likely entry into a viable but nonculturable (VBNC) state of a part of EPS62e population that should cause an underestimation by CFU-counting method, and the presence of nondegraded DNA released from dead cells that should cause an overestimation by Real-time PCR. The combined use of molecular and culture-based methods showed that EPS62e was under optimal colonisation conditions in apple blossoms, and that it was presumably stressed and poorly survived in apple leaves.

The epiphytic fitness of EPS62e on apple and pear was evaluated in orchards from Girona (Spain) during springtime 2005. Several factors affecting the environmental fate of the biocontrol agent EPS62e and its efficacy after field release were evaluated, such as the influence of weather conditions and host species, the influence of indigenous microbiota, and the ability to spread from treated to nontreated trees. Any remarkable difference was observed between the colonisation pattern and population levels in apple and pear blossoms. The biocontrol agent colonised successfully flowers belonging to both species, with high and stable population levels, as observed in preliminary studies in France, reaching the carrying capacity of flowers at 10^7 - 10^8 CFU/blossoms. The weather conditions were supposed to be optimal, since any sharply change was observed on population levels during the assay. EPS62e was able to colonise and dominate the microhabitat of blossoms, representing up to 100% of the cultivable microbiota recovered few days after treatment. Moreover, EPS62e was detected in all nontreated blossoms sampled in trees located 15-35 m far from the inoculated site, showing reliable dispersal ability. Nevertheless, EPS62e population on apple leaves under field conditions decreased during time until it was under the detection threshold of the method 30 days after treatment.

The present work shows that EPS62e is well adapted for blossom colonisation in the field, and encourages its utilisation in a fire blight biological control management.

RESUM

Erwinia amylovora és un bacteri fitopatogen que causa importants pèrdues econòmiques en la producció de pera i poma a nivell mundial. Tradicionalment, el control del foc bacterià s'ha dut a terme mitjançant productes químics, basats majoritàriament en compostos de coure i determinats antibiòtics depenent de la legislació de cada país. No obstant, l'ús reiterat de plaguicides químics ha causat efectes no desitjats com l'aparició de soques resistents i la presència de residus químics en els aliments i el medi ambient. Per aquest motiu, s'han desenvolupat mètodes alternatius de control com el control biològic. Al grup de Patologia Vegetal de la Universitat de Girona es va seleccionar i s'està desenvolupant l'agent de control biològic del foc bacterià *Pseudomonas fluorescens* EPS62e per la seva alta eficàcia en el control de les infeccions causades per *E. amylovora* en fruits immadurs, flors i plantes de perera.

Amb l'objectiu de contribuir al desenvolupament de l'agent de biocontrol *P. fluorescens* EPS62e, en aquest treball es va dissenyar un mètode de traçabilitat molecular basat en la PCR a temps real i es va avaluar la colonització i supervivència de la soca EPS62e després de la seva inoculació a pomeres i pereres a camp.

El desenvolupament d'un mètode de PCR a temps real va comportar (i) la detecció de polimorfismes en el genoma d'EPS62e, (ii) la selecció dels fragments d'amplificació que discriminessin EPS62e en front d'altres soques per fer el disseny d'encebadors SCAR (Sequence Characterised Amplified Regions), (iii) l'avaluació de l'especificitat dels encebadors SCAR, i (iv) el disseny d'una PCR a temps real en una regió interna de la seqüència SCAR. Per a la detecció de seqüències específiques presents de manera natural en el genoma d'EPS62e es van utilitzar dues tècniques basades en la PCR que no requereixen un coneixement previ del genoma, l'amplificació múltiple arbitrària (RAPD) i la PCR inespecífica (U-PCR). Mitjançant ambdues tècniques es van obtenir perfils d'amplificació que permetien la discriminació de EPS62e d'altres soques de la mateixa espècie. Es van seqüenciar tres fragments d'amplificació específics de l'EPS62e obtinguts mitjançant RAPD i U-PCR i es van dissenyar una parella d'encebadors per cada seqüència per obtenir marcadors SCAR. L'especificitat dels encebadors SCAR es va avaluar en front 161 soques pertanyents a la mateixa espècie, 75 soques pertanyents a espècies relacionades i 61 mostres vegetals procedents de finques comercials. De les tres parelles d'encebadors SCAR dissenyades, dues parelles varen ser totalment específiques per la detecció de EPS62e, mentre que la tercera parella d'encebadors SCAR va mostrar no ser específica de l'EPS62e ja que es van obtenir amplificacions en altres soques de la mateixa espècie. A partir dels dos marcadors SCAR específics es van dissenyar dues PCR a temps real mitjançant sondes tipus TaqMan®. Els dos dissenys de PCR a temps real varen ser específics per EPS62e, amb un nivell de detecció de 10^2 UFC/g pes fresc i una corba estàndard linear en un rang de cinc unitats logarítmiques.

Durant el desenvolupament i l'optimització de la PCR a temps real, la traçabilitat de la població cultivable d'EPS62e a fulles de perera i en condicions d'ambient controlat es va dur a terme mitjançant dues tècniques clàssiques acoblades a PCR. Aquestes tècniques es basaren en el recompte de viables en placa (RVP) i el nombre més probable (NMP) en medi selectiu, seguides d'una PCR amb els encebadors SCAR. Ambdues tècniques varen ser útils per la traçabilitat de EPS62e, ja que no es varen observar diferències significatives entre els valors estimats per cadascuna. El nivell poblacional cultivable de EPS62e va disminuir progressivament des de 10^7 fins a 10^5 – 10^4 UFC/g pes fresc durant els primers 17 dies, i després es va mantenir estable durant 11 dies més fins al final de l'assaig. Malgrat la idoneïtat d'ambdues tècniques per la traçabilitat de EPS62e, es va seleccionar el RVP en medi selectiu pels següents assajos.

La PCR a temps real es va validar com a eina per la traçabilitat de EPS62e mitjançant la seva utilització simultània amb el mètode de RVP en diversos experiments en flors i fulles de pomera, i els resultats es van comparar i contrastar estadísticament. Els experiments es van dur a terme en condicions d'hivernacle i camp a la regió de la Maine-et-Loire (France) durant la primavera del 2004. En els resultats obtinguts amb flors de pomera es va observar una bona correlació dels nivells poblacionals estimats per PCR a temps real i RVP. L'agent de biocontrol EPS62e va ser un colonitzador eficient de flors de pomera, assolint alts nivells poblacionals que oscil·laven entre 10^7 i 10^8 UFC/corimbe. Aquesta població es va mantenir fins i tot durant la formació i el creixement del fruit, 54 dies després del tractament, on fins al 99% de la població es trobava restringida a l'àrea del calze del fruit. D'altra banda, en els experiments duts a terme en fulles de pomera la situació va ser completament diferent, ja que els nivells poblacionals d'EPS62e van disminuir al llarg del temps. En fulles de pomera, els valors estimats per PCR a temps real van ser significativament superiors als obtinguts pel RVP. Per explicar la diferència entre mètodes observada es va concloure que hi havia dos possibles factors involucrats: l'entrada a un estat viable però no cultivable (VBNC) d'una part de la població d'EPS62e que podria haver causat una subestimació mitjançant el RVP, i la presència de DNA no degradat alliberat de cèl·lules mortes que podria haver causat una sobreestimació per PCR a temps real. L'ús combinat de mètodes moleculars i cultiu-dependents va mostrar que EPS62e es trobava sota condicions òptimes de colonització a les flors de pomera, i que estava presumiblement sota estrès i sobrevivia amb dificultats a les fulles de pomera.

L'adaptabilitat epifítica d'EPS62e en pomeres i pereres es va avaluar en condicions de camp a Girona durant la primavera del 2005. S'avaluaren diferents factors que podien afectar el seu esdevenir i la seva eficàcia ecològica després de ser alliberada a camp, com la influència de les condicions climàtiques, l'espècie de planta hoste, la influència de la microbiota autòctona i l'habilitat de dispersar-se des dels arbres tractats fins als no tractats. No es va observar cap diferència remarcable entre el patró de colonització i els nivells poblacionals de EPS62e en flors de pomera i perera. L'agent de biocontrol va colonitzar satisfactòriament les flors pertanyents a ambdues espècies, amb elevats nivells poblacionals tal i com s'havia observat en estudis anteriors realitzats a França, assolint la capacitat de càrrega microbiana de les flors a 10^7 - 10^8 UFC/corimbe. Les condicions climàtiques semblaren ser òptimes ja que no es va observar cap canvi brusc en els nivells poblacionals durant l'assaig. EPS62e va ser capaç de colonitzar i dominar el microhabitat de les flors, representant fins al 100% de la microbiota bacteriana cultivable total pocs dies després del tractament. A més, EPS62e es va detectar a totes les flors no tractades allunyades entre 15 i 35 m del lloc d'inoculació, mostrant una bona habilitat de dispersió.

Tanmateix, la població de EPS62e en fulles de pomera sota condicions de camp va disminuir al llarg del temps fins a estar sota el límit de detecció de la tècnica 30 dies després del tractament.

Aquest treball mostra que la soca EPS62e està ben adaptada a la colonització de flors a camp, encoratjant la seva utilització dins d'una estratègia de control biològic contra el foc bacterià.

RESUMEN

Erwinia amylovora es una bacteria fitopatógena que causa importantes pérdidas económicas en la producción de pera y manzana a nivel mundial. Tradicionalmente, el control del fuego bacteriano se ha realizado mediante productos químicos, formulados mayoritariamente con compuestos de cobre o antibióticos dependiendo de la legislación en cada país. No obstante, el uso reiterado de plaguicidas químicos ha causado efectos indeseables como la aparición de cepas resistentes y la presencia de residuos en los alimentos y el medio ambiente. Por este motivo, se han desarrollado métodos alternativos de control como el control biológico. En el grupo de Patología Vegetal de la Universidad de Girona se seleccionó y se está desarrollando el agente de control biológico del fuego bacteriano *Pseudomonas fluorescens* EPS62e por su alta eficacia en el control de infecciones causadas por *E. amylovora* en frutos inmaduros, flores y plantas de peral.

Con el objetivo de contribuir al desarrollo del agente de biocontrol *P. fluorescens* EPS62e, en este trabajo se diseñó un método de trazabilidad molecular basado en la PCR a tiempo real y se evaluó la colonización y supervivencia de la cepa EPS62e después de su inoculación en manzanos y perales en campo.

El desarrollo de un método de PCR a tiempo real implicó (i) la detección de polimorfismos en el genoma de EPS62e, (ii) la selección de fragmentos de amplificación que discriminasen EPS62e frente a otras cepas para el diseño de cebadores SCAR (Sequence Characterised Amplified Regions), (iii) la evaluación de la especificidad de los cebadores SCAR, y (iv) el diseño de una PCR a tiempo real en una región interna de la secuencia SCAR. Para la detección de secuencias específicas presentes de manera natural en el genoma de EPS62e se utilizaron dos métodos basados en la PCR que no requieren un conocimiento previo del genoma, la amplificación múltiple arbitraria (RAPD) y la PCR inespecífica (U-PCR). Mediante ambas técnicas se obtuvieron perfiles de amplificación que permitieron la discriminación de EPS62e frente a otras cepas de la misma especie. Se secuenciaron tres fragmentos de amplificación específica de EPS62e obtenidos mediante RAPD y U-PCR y se diseñaron un par de cebadores en cada secuencia para la obtención de marcadores SCAR. La especificidad de los cebadores SCAR se evaluó frente a 161 cepas pertenecientes a la misma especie, 75 cepas pertenecientes a especies relacionadas y 61 muestras vegetales de fincas comerciales. De los tres pares de cebadores SCAR diseñados, dos parejas fueron totalmente específicas para la detección de EPS62e, mientras que el tercer par de cebadores SCAR mostró no ser específico ya que se obtuvieron amplificaciones en otras cepas de la misma especie. A partir de los dos marcadores SCAR específicos se diseñaron dos PCR a tiempo real mediante sondas de tipo TaqMan®. Los dos diseños de PCR a tiempo real fueron específicos para EPS62e, con un nivel de detección de 10^2 UFC/g peso fresco y una curva estándar linear en un rango de cinco unidades logarítmicas.

Durante el desarrollo y optimización de la PCR a tiempo real se realizó un estudio de trazabilidad de la población cultivable de EPS62e, en hojas de peral en condiciones de ambiente controlado, mediante dos técnicas clásicas acopladas a PCR. Las dos técnicas se basaron en el recuento de viables en placa (RVP) y el número más probable (NMP) en medio selectivo, seguidas de una PCR con los cebadores SCAR. Ambas técnicas fueron útiles para la trazabilidad de EPS62e, ya que no se observaron diferencias significativas entre los valores estimados por las dos técnicas. El nivel poblacional cultivable de EPS62e disminuyó progresivamente desde 10^7 hasta 10^5 – 10^4 UFC/g peso fresco durante los primeros 17 días, y después se mantuvo estable durante 11 días más hasta el fin del ensayo. A pesar de la idoneidad de ambas técnicas para la trazabilidad de EPS62e, se seleccionó el RVP en medio selectivo para los siguientes ensayos.

La PCR a tiempo real se validó cómo método de trazabilidad de EPS62e mediante su uso simultáneo con el método de RVP en diversos experimentos en flores y hojas de manzano, y los resultados se compararon y contrastaron estadísticamente. Los experimentos se llevaron a cabo en condiciones de invernadero y campo en la región de Maine-et-Loire (Francia) durante la primavera del año 2004. Los resultados obtenidos con flores de manzano mostraron una buena correlación de los niveles poblacionales estimados por PCR a tiempo real y RVP. El agente de biocontrol EPS62e fue un colonizador eficiente de flores de manzano, alcanzando niveles poblacionales altos que oscilan entre 10^7 y 10^8 UFC/corimbo. Esta población se mantuvo durante el cuajado y el desarrollo del fruto, 54 días después del tratamiento, donde hasta el 99% de la población estaba restringida en el área del cáliz del fruto. En cambio, los resultados obtenidos con hojas de manzano fueron completamente diferentes, ya que los niveles poblacionales de EPS62e disminuyeron a lo largo del tiempo. En hojas de manzano, los valores estimados por PCR a tiempo real fueron significativamente diferentes a los valores obtenidos por el RVP. En general, los valores de PCR a tiempo real fueron superiores a los valores de RVP. Esta diferencia entre métodos se atribuyó a dos posibles causas: la entrada en un estado viable pero no cultivable (VBNC) de una parte de la población de EPS62e pudiendo causar una subestimación mediante el RVP, y a la presencia de DNA no degradado procedente de células muertas pudiendo causar una sobreestimación por PCR a tiempo real. El uso combinado de métodos moleculares y cultivo-dependientes mostró que EPS62e se encontraba bajo condiciones óptimas de colonización en flores de manzano y que estaba presumiblemente bajo estrés y sobrevivía con dificultad en hojas de manzano.

La adaptabilidad epifítica de EPS62e en manzanos y perales se evaluó en condiciones de campo en Girona durante la primavera del año 2005. Se evaluaron diferentes factores que pueden afectar su devenir y su eficacia ecológica después de ser liberada en campo, como la influencia de las condiciones climáticas, la especie de planta huésped, la influencia de la microbiota autóctona, y su habilidad de dispersión desde árboles tratados hasta árboles no tratados. No se observó ninguna diferencia remarcable entre el patrón de colonización y los niveles poblacionales de EPS62e en flores de manzano y peral. El agente de biocontrol colonizó satisfactoriamente las flores pertenecientes a ambas especies, con niveles poblacionales elevados como se había observado en estudios anteriores realizados en Francia, alcanzando la capacidad de carga microbiana de las flores a 10^7 – 10^8 UFC/corimbo. Las condiciones climáticas parecieron ser óptimas ya que no se observó ningún cambio brusco en los niveles poblacionales durante los ensayos. EPS62e fue capaz de colonizar y dominar el microhabitat de las flores, representando hasta el 100% de la microbiota cultivable total

pocos días después del tratamiento. Además, EPS62e fue detectada en todas las flores no tratadas situadas entre 15 y 35 m del lugar de inoculación, mostrando una buena habilidad de dispersión. Aun así, la población de EPS62e en hojas de manzano bajo condiciones de campo disminuyó a lo largo del tiempo hasta estar por debajo del límite de detección de la técnica 30 días después del tratamiento.

Este trabajo muestra que la cepa EPS62e está bien adaptada a la colonización de flores en campo, alentando su utilización en una estrategia de control biológico del fuego bacteriano.

RESUMÉ

Erwinia amylovora est une bactérie phythopathogène qui cause d'importantes pertes économiques dans la production de poires et de pommes au niveau mondial. Traditionnellement, la lutte chimique, basée sur l'utilisation des composés du cuivre ou des antibiotiques selon la législation de chaque pays, a été la principale mesure de contrôle du feu bactérien. Néanmoins, l'utilisation constante des produits chimiques a conduit à l'apparition de phénomènes de résistance chez le pathogène ainsi que à la présence de résidus sur les denrées alimentaires et dans l'environnement. En vue de surmonter ces inconvénients, d'autres moyens de lutte ont été développés, comme la lutte biologique. Dans ce contexte, la souche EPS62e, appartenant à l'espèce *Pseudomonas fluorescens,* a été sélectionné par l'Unité de Phytopathologie de l'Université de Girona. Cette souche est en cours de développement en tant qu'agent de lutte biologique du feu bactérien, car elle confère une haute efficacité dans le contrôle des infections causées par *E. amylovora* sur des fruits verts, des fleurs et des plants de poirier.

Afin de contribuer au développement de l'agent de lutte biologique *P. fluorescens* EPS62e, une méthode de traçage moléculaire par PCR en temps réel a été mise au point au cours de ce travail. Elle a été utilisée afin d'évaluer la colonisation et la survie de la souche EPS62e après son application sur pommiers et poiriers au verger.

La mise au point d'une technique de PCR en temps réel a impliqué (i) la détection de polymorphismes dans le génome de la souche EPS62e, (ii) l'utilisation de ce polymorphisme pour sélectionner des amorces spécifiques de type SCAR (Sequence Characterised Amplified Region), (iii) l'évaluation de la spécificité des amorces SCAR, et (iv) le design des amorces et d'une sonde pour la PCR en temps réel dans une région interne de la séquence SCAR. Pour l'identification de marqueurs naturels chez le génome de la souche EPS62e, deux techniques basées sur la PCR, soit l'amplification aléatoire de l'ADN polymorphe (RAPD) et la PCR non-spécifique (U-PCR), ont été utilisées. Ces deux techniques présentent l'avantage de pouvoir identifier des marqueurs sans aucune connaissance préalable du génome de la souche ciblée. Les deux techniques ont mis en évidence des profils différentiels pour la souche EPS62e vis-à-vis d'autres souches de l'espèce. Trois fragments différentiels obtenus par RAPD et U-PCR ont été séquencés et caractérisés comme marqueurs SCAR. A partir de ces séquences, trois couples d'amorces SCAR ont été sélectionnés, et leur spécificité a été évaluée sur 161 souches appartenant à la même espèce, sur 75 souches appartenant à des espèces proches et sur 61 échantillons végétaux provenant de vergers. Parmi les trois couples d'amorces SCAR développés, deux couples d'amorces ont été complètement spécifiques pour la détection de la souche EPS62e, tandis que la troisième couple d'amorces a amplifié d'autres souches de la même espèce. À partir des marqueurs SCAR spécifiques, deux PCR en temps réel ont été développées avec sondes de type TaqMan®. Les deux protocoles de PCR en temps réel ont été spécifiques à la souche EPS62e, avec un seuil de détection de 10^2 UFC/g poids frais et une courbe standard linéaire sur cinq unités logarithmiques.

Pendant le développement et l'optimisation de la PCR en temps réel, le traçage de la population cultivable de EPS62e à la surface des feuilles de poirier dans des conditions d'environnement contrôlé a été réalisé selon deux méthodes classiques. Ces méthodes étaient basées sur le comptage des cellules viables sur boite de pétri (CVB) et sur le nombre le plus probable (NPP), suivies d'une PCR avec les amorces SCAR. Les deux méthodes ont été comparées et elles ont été jugées appropriées pour le traçage de la souche EPS62e. En effet, aucune différence significative entre les résultats obtenus par une ou l'autre méthode n'a été observée. Le niveau de la population cultivable de EPS62e a diminué progressivement de 10^7 à 10^5-10^4 UFC/g poids frais pendant les 17 premiers jours. Il est ensuite resté stable pendant 11 jours jusqu'à la fin de l'essai. Bien

que ces deux techniques se soient révélées performantes, seul le CVB dans du milieu sélectif a été sélectionné pour les essais ultérieurs.

L'utilisation de la PCR en temps réel pour le traçage de la souche EPS62e à été validée par comparaison de ses résultats avec ceux obtenus par la méthode du CVB dans plusieurs expériences sur fleurs et feuilles de pommier. Les expériences ont été menées en serre et au verger dans le département du Maine-et-Loire (France) pendant le printemps 2004. Dans les essais réalisés sur fleurs de pommier, le niveau de la population estimé par PCR en temps réel et par CVB a montré une bonne corrélation. L'agent de lutte biologique EPS62e a montré son pouvoir de colonisation des fleurs de pommier en serre et au verger, car il a atteint un haut niveau de population entre 10^7 et 10^8 UFC/corymbe. Cette population est restée stable pendant la formation et la croissance des fruits, 54 jours après le traitement, où 99% de la population était limitée à la région du calice du fruit. A contrario, la population de EPS62e dans les feuilles de pommier a diminué avec le temps. Sur les feuilles, les valeurs obtenues par PCR en temps réel et CVB ont été significativement différentes. En général, les estimations de la PCR en temps réel ont été supérieures aux valeurs de cultivables. La différence observée a été expliquée par deux facteurs principalement : (1) l'existence de cellules viables mais non-cultivables (VBNC) au sein de la population de EPS62e (sous-estimation par la méthode du CVB) et (2) la présence d'ADN non-dégradé issu de cellules mortes (surestimation par la PCR en temps réel). L'utilisation combinée des méthodes moléculaires et microbiologiques a montré que la souche EPS62e était dans des conditions optimales de colonisation sur fleurs de pommier. D'autre part, elle était probablement stressée et pouvait difficilement survivre sur feuilles de pommier.

Finalement, pendant le printemps 2005, l'adaptation épiphytique de la souche EPS62e sur pommiers et poiriers à Girona (Espagne) a été évaluée. Plusieurs facteurs pouvant affecter le comportement écologique de la souche ont été étudiés. Il s'agissait de l'influence des conditions climatiques, de l'espèce de plante hôte et de la microflore autochtone ainsi que la capacité de dispersion de la souche depuis les arbres traités vers les arbres non traités. Aucune différence n'a été observée entre la colonisation des fleurs de pommier et de poirier. L'agent de lutte biologique a colonisé d'une façon satisfaisante les fleurs appartenant aux deux espèces, avec un niveau de population stable et élevée, confirmant ainsi les études préliminaires menées en France. Les conditions climatiques on été probablement optimales, car il n'y a pas eu aucun changement notable au niveau de la population pendant l'essai. EPS62e a été capable de coloniser les fleurs et d'en dominer la microflore. En effet, quelque jours après le traitement, elle a représenté jusqu'au 100% de la microflore cultivable total. En plus, EPS62e a été détectée sur toutes les fleurs non traitées situées entre 15 et 35 m du point d'inoculation. Elle a ainsi montré

une bonne habilité de dispersion. Cependant, la population de EPS62e sur feuilles de pommier au verger a diminué avec le temps jusqu'à un niveau non détectable 30 jours après traitement.

Ce travail montre que la souche EPS62e est très adaptée à la colonisation des fleurs au verger, ce qui encourage son utilisation dans une stratégie de lutte biologique contre le feu bactérien.

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LIST OF ABREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
CFU	Colony Forming Unit
ERIC	Enterobacterial Repetitive Intergenic Consesnus
FCM	Flow cytometry
fw	Fresh weight
GFP	Green Fluorescent Protein
GMM	Genetically Modified Microorganism
ITS	Internal Transcribed Spacer
LUX	Luciferase Enzyme
MPN	Most Probable Number
MRFLP	Macrorestriction Fragment Length Polymorphism
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
QC-PCR	Quantitative-Competitive Polymerase Chain Reaction
Q-PCR	Quantitative-Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
rep-PCR	Repetitive Extragenic Palindromic-Polymerase Chain Reaction
SCAR	Sequence Characterised Amplified Region
U-PCR	Unspecific-Polymerase Chain Reaction
VBNC	Viable But Nonculturable

INTRODUCTION

FIRE BLIGHT, THE DISEASE

Fire blight is a systemic bacterial disease that affects several plant species, mainly belonging to the subfamily *Maloideae* of the rosaceous family, e.g. fruit trees such as pear (*Pyrus* spp.) and apple (*Malus* spp.) and ornamentals such as *Cotoneaster* spp., *Crataegus* spp. and *Pyracantha* spp; and also to some species belonging to the subfamily *Amygdaloideae*, e.g. Japanese plum (*Prunus salicina*), and *Rosoideae*, e.g. raspberry bush (*Rubus idaeus*) (Van der Zwet & Beer, 1995). The causative agent is *Erwinia amylovora* (Burrill, 1883; Winslow *et al*., 1920), a Gram-negative bacterium belonging to the Enterobacteriaceae family. *E. amylovora* is a necrogenic bacterium that once it infects the plant, it moves rapidly through the intercellular spaces of the parenchyma and in the xylem vessels, causing severe lesions in the vascular system (Goodman & White, 1981). Its ability to progress in host plant tissues can lead, under favourable conditions, the pass

of the pathogen from the flowers to the woody tissue, being able to kill the tree within a single growing season (Vanneste & Eden-Green, 2000). For this reason, it is not surprising that fire blight has been described as one of the most devastating bacterial diseases of pomaceous fruit trees (Van der Zwet, 2002). The economical losses are difficult to be determined, however, in the worst cases after severe outbreaks, the orchard productions are disrupted for years (Vanneste, 2000).

The disease is named after its **symptoms**, since the pathogen blackens the leaves, flowers, shoots and even the whole tree (Figure 1). The most susceptible organs are flowers and young shoots. The first symptoms usually appear in blossoms, where drops of bacterial ooze are present in the nectarial cup. When the disease progresses, exudates can be observed down to the pedicel and then, flower clusters turn brown and die. In young shoots, the first symptoms are drops of exudates on the entire length of the still turgescent shoot, whereas old infections are characterised by wilted and necrosed tissues. The infected growing shoots often exhibit a typical curling at the end, called a shepherd's crook. Depending on the host species, the symptoms may slightly change, such as the colour of the necrosed tissue, changing from dark brown to black in pear trees and from red to dark brown in apple trees. Blighted leaves remain attached to the tree through much of the dormant season. The fruit may also become infected, turning brown and presenting droplets of ooze, remaining attached through the winter on a mummified appearance. Bacterial cankers can be formed when the infection progresses into the woody tissue, with a discoloration of the bark.

The comprehension of the **disease cycle** is the basis for the understanding of its worldwide spread (Figure 2). The source of primary inoculum comes from cankers, which are infected plant tissues where the bacterium has overwintered and survived without producing external symptoms. During springtime, *E. amylovora* can be disseminated from cankers to flowers by insects, wind and rain. *E. amylovora* grows epiphytically on the stigmatic surfaces of flowers, where its population multiplies rapidly. During its multiplication in flowers, the high population level becomes a source of secondary inoculum, being disseminated to other tissues or trees by insects, wind or rain. The pathogen moves from the stigmatic surfaces to the nectharodes, which are the main pathway to enter into the host plant tissues. Stigmas, anthers and stomata are other natural openings also used by *E. amylovora* to infect the plant. The pathogen can also penetrate the plant by wounds, e.g. on leaf tissues or immature fruits. Once *E. amylovora* penetrates the plant, it multiplies into intercellular spaces and moves rapidly through the cortical parenchyma, the phloem and the xylem vessels, infecting shoots. These infections can progress into the woody tissue producing cankers, providing the overwintering survival place.

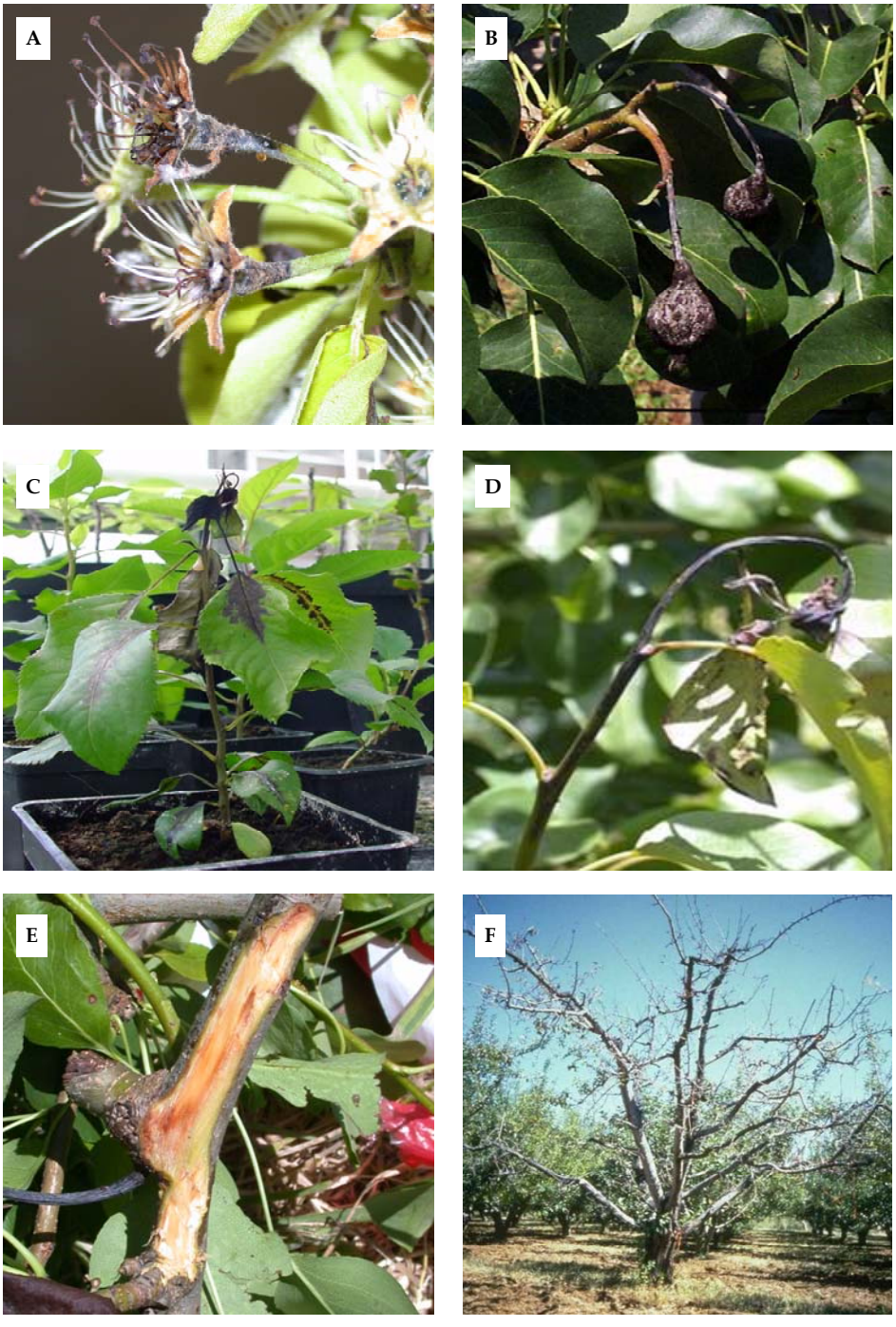

Figure 1: Fire blight symptoms. (A) Blacken flowers with exudates in the pedicel; (B) infected and mummified fruit; (C) droplets of ooze of an infected nursery; (D) infected growing shoot showing the shepherd's crook symptom; (E) bacterial canker in woody tissue; (F) killed tree by *E. amylovora* infection. (Juan, 1999; Ruz, 2003; Cabrefiga, 2004).

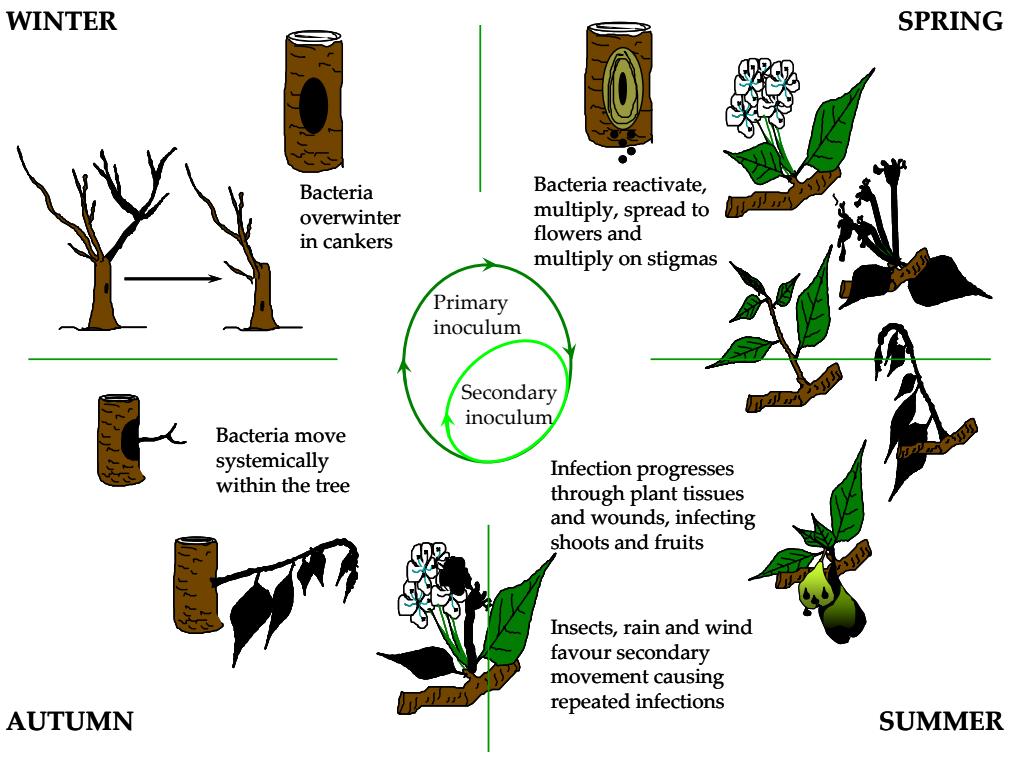

Figure 2: Disease cycle of fire blight (Montesinos & López, 1998)

The **geographical distribution** of fire blight has a long history that started with its first observation in 1780 in Hudson Valley, New York, USA (Denning W., 1794). Since then, it has not stopped to spread, affecting nowadays about 42 countries around the world (Figure 3) (Van der Zwet, 2002). From New York, the disease spread through North America to the Pacific Rim. In 1919 fire blight was first detected in New Zealand. The pathogen entered in Europe during the 1950s in two different locations: one in northwestern Europe and the other in the north-east corner of Africa (Figure 3B). During the next 30 years, the disease spread through several European and Middle East countries.

In Spain, fire blight was first detected in 1995 in Guipuzkoa (Euskadi), close to the Antlantic French border (Butrón, 1995; De la Cruz Blanco, 1996; López *et al*., 1996) (Figure 3C). Since then, the most affected regions have been Guipuzkoa and Navarra, situated in the northern Spain. However, other single outbreaks have been detected and eradicated in Segovia (1996), Guadalajara (1998), Madrid (1998), Lleida and Huesca

(1998, 1999), and in La Rioja and Zaragoza (from 2000 to 2002) (Montesinos & López, 1998; Montesinos *et al*., 1999; Montesinos & Llorente, 1999; López *et al*., 2002; Donat *et al*., 2005). The last outbreak was detected and eradicated in Girona (2003).

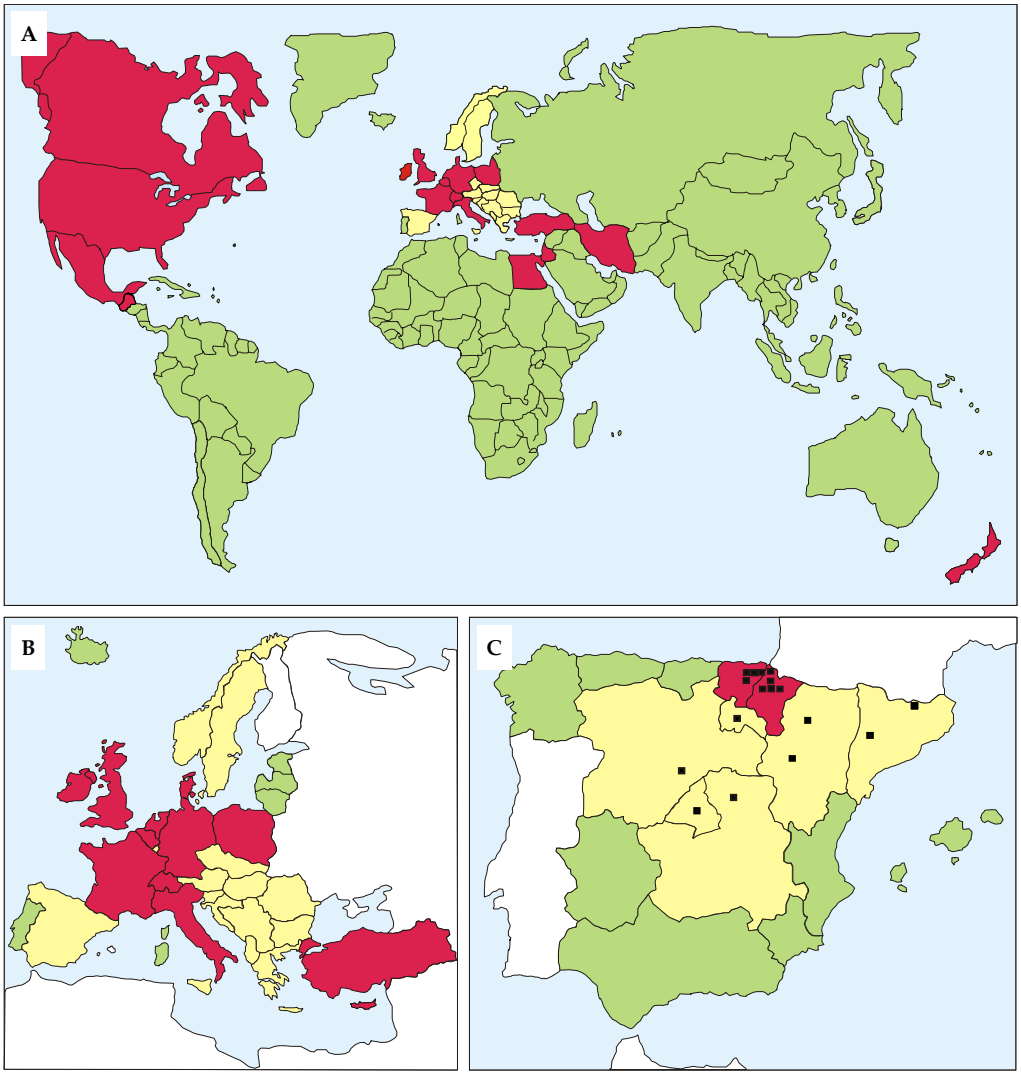

Figure 3: Worldwide distribution of fire blight (Ruz, 2003) based on data reviewed by EPPO (2005-03) and Donat *et al.* (2005). ■ Countries or regions where fire blight is widely and early distributed, □ where is partially distributed, ■ and where has still not been described. ■ Fire blight outbreaks in Spain, from 1995 to 2005.

Fire blight is considered a quarantine disease in the European Union, so it was included in the Council Directive 77/93/EEC, amended by the Council Directive 2000/29/EC, on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. In consequence, a phytosanitary passport was regulated and standardised by the Comission Directive 92/105/EEC, amended by the Comission Directive 2005/17/EC, to assure that plants comply with regulations concerning organisms that lead to quarantine, and which allows them to be transported freely in the European Union. Under these regulations, Spain is considered a protected zone against fire blight, thus there is a legislation to control any signal of the presence of the pathogen along the country (law 43/2002 of 20th November of Plant Health).

The **management** of fire blight is complex, since there is not any single treatment able to control the disease after its appearance, i.e. all treatments are preventive, not curative. Therefore, almost all control strategies aim to prevent the arrival and the progress of the pathogen into the host plant. This arrival is controlled at a country level, by means of import barriers for the plant hosts of fire blight to avoid the introduction of contaminated plants; and at a field level, by eradicating contaminated and non-contaminated plants in a defined area round the detected outbreak. For a rapid detection of *E. amylovora*, several biochemical and molecular methods have been developed, such as the immunological detection by means of ELISA-DASI method (Gorris *et al*., 1996), the PCR detection methods based on plasmid pEA29 (Bereswill *et al*., 1992), chromosomal DNA (Bereswill *et al*., 1995), 23s rDNA (Maes *et al*., 1996), nested PCR (Llop *et al*., 2000), the Real-time PCR directed to the plasmid pEA29 (Salm & Geider, 2004), and finally the DNA macroarray system based on rDNA sequences (Sholberg *et al*., 2005). Moreover, other molecular methods have been developed to characterise the diversity of isolated strains of *E. amylovora* species and to study the epidemiology of the disease, such as pulsed field gel electrophoresis (PFGE) of restriction fragment length polymorphisms (RFLPs) (Zhang & Geider, 1997; Jock *et al*., 2002) and amplified fragment length polymorphism (AFLP) (Rico *et al*., 2004) methods.

Traditionally, the most commonly used control strategy for fire blight management has been chemical control. Chemical pesticides aim to render plant surfaces unsuitable for pathogen colonisation or to eliminate the pathogen before it penetrates the host tissue (Psallidas & Tsiantos, 2000). We have to differentiate between bactericide compounds, such as copper compounds (Bordeaux mixture, copper oxychloride, ammonium copper sulphate, copper hydroxide, copper oxide and mixtures of copper compounds with maneb, zineb and oil) which have not a systemic action, from others such as antibiotics (streptomycin, oxytetracycline, kasugamycin) restricted to some

countries. Other chemical compounds have been developed to minimise the phytotoxicity and resistance problems caused by copper compounds and antibiotics, such as fosetyl-aluminium (Aliette®), harpin (Messenger®), prohexadione-Ca (Apogee®) or benzothiadiazole (Bion®, Actigard®). These compounds induce acquired systemic resistance in the host plant or are plant growth regulators that lower plant susceptibility to *E. amylovora* infection.

The application of chemical pesticides can cause phytotoxicity, so their use must be in accordance to plant susceptibility, being the recommended doses lower during bloom than during dormancy. The optimised application must also be related to the maximum risk of disease to obtain the highest efficacy. This risk can be assessed by using forecasting models such as Maryblyt (Steiner P.W., 1990) and Cougarblight (Smith, 1993).

The main drawback concerning the use of chemical pesticides is the development of pathogen resistance, as it has been observed in several countries after the repetitive use of certain antibiotics such as streptomycin (Moller *et al*., 1981; Loper *et al*., 1991; McManus *et al*., 2002). Moreover, the antibiotics commercialised and used in the United States to control fire blight are banned in Europe. Copper compounds are also under study to prevent the appearance of resistances in *E. amylovora* strains, as it has been observed for other plant pathogenic bacteria such as *Xanthomonas campestris* pv. vesicatoria and *Pseudomonas syringae* pv. tomato (Sundin *et al*., 1989; Cooksey, 1990). Other drawbacks in fire blight chemical control are the environmental impact on soil and groundwater after a reiterative use of chemicals, and the presence of chemical residues in food.

Social concerns about safety in agriculture products and new policies have favoured the research and development of alternative methods that involve an Integrated Pest Management program, which only uses the chemical strategy at the last level, when there is no other alternative control strategy (McSpadden Gardener & Fravel, 2002). These policies resulted in the European Directive 91/414/EEC which aimed to assure the safety for humans and environment of the authorised plant protection products that are commercialised in the European market. These social and political changes supporting new strategies of plant disease control improved the development of biological control strategies, and the Council Directive 91/414/EEC was amended by the Commission Directive 2001/36/EC for adapting the legislation to the use of other kind of active ingredients for plant protection such as microorganisms.

According to the definition of the International Organisation for Biological and Integrated Control of Noxious Animals and Plants (IOBC), the biological control can be

defined as the use of an organism to reduce the population density of another organism, or even simpler, using biota to reduce biota. Biological control has to be considered a complementary method in an integrated pest management of fire blight, mainly focused in the prevention of fire blight disease during bloom. The efficiency of biological control will be increased by using additional measures such as the maintenance of the sanitation of the orchard removing any source of overwintering inoculum, and risk assessment by using forecasting models.

BIOLOGICAL CONTROL OF FIRE BLIGHT

In the last 25 years, several studies have been done for the development of reliable biological control strategies for fire blight management. For this purpose, several strains have been selected and studied because of their ability to prevent or suppress the progress of the disease, some of which are currently available for orchardists, and others are in process of approval of their commercialisation in different countries (Table 1). The current biocontrol agents of fire blight mainly belong to three species: *B. subtilis*, *P. agglomerans*, and *P. fluorescens*, and the most studied mechanisms involved in biocontrol are antibiosis and competitive exclusion.

The key point to biocontrol *E. amylovora* infections is based on the suppression of the pathogen in blossoms, since the pathogen needs to multiply on the stigmatic surfaces prior to penetrate and infect the plant (Johnson & Stockwell, 1998). If the multiplication of *E. amylovora* is inhibited, consequently, the probability of blossom infection and the dispersal of *E. amylovora* to other blossoms will be reduced.

Table 1: Biological control agents of fire blight

Organism	Mode of action	Commercial name (company)	Source
Bacillus pumilus QST2808	Antibiosis	Sonata® (AgraQuest)	AgraQuest, INC.
Bacillus subtilis BD170	Antibiosis	Biopro® (Andermatt Biocontrol AG)	Broggini *et al*., 2005
B. subtilis QST713	Antibiosis	Serenade® (AgraQuest)	Aldwinckle *et al*., 2002
B. subtilis BS-F3	Antibiosis	BS-F4® (Agribiotec)	Alexandrova *et al*., 2002
Pantoea agglomerans C9-1	Antibiosis	BlightBan C9-1 (Plant Health Technologies)	Ishimaru *et al*., 1988
P. agglomerans D325	Competitive inhibition	Bloomtime Biological FD® (Northwest Agricultural Products)	Pusey, 1997
P. agglomerans E252	Antibiosis	-	Vanneste *et al*., 1992
P. agglomerans Eh24	Competitive exclusion	-	Ozaktan *et al*., 1999
P. agglomerans Eh112Y	Antibiosis	-	Wodzinski *et al*., 1994
P. agglomerans Eh318	Antibiosis	-	Wright & Beer, 1996
P. agglomerans Eh1087	Antibiosis	-	Kearns & Hale, 1996
P. agglomerans EhHI9N13	Antibiosis	-	Wilson *et al*., 1990
P. agglomerans P10c	Competition, antibiosis	Blossom Bless®, PomaVita® (HortResearch, Agrifutur)	Vanneste *et al*., 2002
Pseudomonas fluorescens A506	Competitive exclusion, antibiosis	BlightBan® A506 (NuFarm)	Wilson & Lindow, 1993; Temple *et al*., 2004
P. fluorescens EPS62e	Competitive exclusion	-	Cabrefiga, 2004

The development of a biological control agent involves several steps. Firstly, the selection of effective antagonist strains able to control the pathogen not only in the laboratory, but also under natural disease outbreak conditions. Secondly, the study of the mechanisms of action that provide the capacity to inhibit the pathogen's establishment, growth or plant infection. In parallel, formulation and mass production systems must be developed in order to increase the efficacy of the biocontrol agent and to make its commercialisation feasible. Concerning the commercialisation of biocontrol agents in the European market, a registration procedure is required. This procedure, according to European Directives 91/414/EEC and 2001/36/EC, involves the fulfilment of toxicological and ecotoxicological studies to assure the safety of the new biopesticide against the human health and the environment (Montesinos, 2003; Mark *et al*., 2006). The development of monitoring methods that provide the specific detection and quantification of the biocontrol agent will make feasible the performance of ecotoxicological studies, as well as the evaluation of its ecological fitness and the impact of the formulation and the method of application.

The **selection** of effective antagonist strains has been traditionally made by the evaluation of the isolated strains' capacity to inhibit *E. amylovora* in media-based assays and immature pear fruit assays. These procedures favoured those antagonists which

exhibited antibiosis as a major mechanism of action. However, it did not always correlate with the efficacy of the selected antagonists to inhibit *E. amylovora* in blossoms (Wilson *et al*., 1990; Wilson *et al*., 1992). Therefore, the evaluation of isolated strains according to their capacity to inhibit *E. amylovora* multiplication in blossoms is preferred (Mercier & Lindow, 1996). In this way, procedures have been developed to test antagonist strains in blossoms even during nonseasonal availability of flowers, by forcing bloom under controlled environment (Pusey, 1997). It is recommended to isolate large numbers of bacteria that naturally colonise the target organ of the host plant, and then screen among them concerning its antagonist effect; without a prior selection based on their mode of action (Cook & Baker, 1983; Montesinos, 2003; Fravel, 2005). These bacteria will presumably be better adapted to *in situ* ecological stresses than bacteria selected from other environments.

Once the antagonistic strain is selected, it is necessary to comprehend the mechanism involved in the biocontrol activity, despite the complexity of this kind of studies that involves the biocontrol agent, the pathogen, the host plant, the environment and the indigenous microbiota. The **mechanisms of action** of the currently existing biological control agents of fire blight can be divided into two groups. The first group is mainly formed by strains belonging to *P. fluorescens* species (Wilson & Lindow, 1993; Cabrefiga, 2004), which are characterised for their ability to colonise stigmatic surfaces up to their carrying capacity (Wilson *et al*., 1992; Wilson & Lindow, 1993). The arrival of these strains before the pathogen causes a competitive exclusion, so the pathogen does not find sites to colonise or nutrients available. The second group is formed by strains belonging to *P. agglomerans*, *B. subtilis* and *B. pumilus* species. These strains produce one or more antimicrobial substances which inhibit the growth of the pathogen. The antimicrobial activity has been easily demonstrated *in vitro*; however, other complex studies have been carried out to show the contribution of antibiosis in the orchard, as in the case of the biocontrol strain *P. agglomerans* E252 (Stockwell *et al*., 2002). Despite the above mentioned two types of groups, it has been observed that various mechanisms are commonly involved, thus antimicrobial activity is usually coupled to a competition for sites and nutrients too (Kearns & Hale, 1996; Wright & Beer, 1996; Stockwell *et al*., 2002; Temple *et al*., 2004). Therefore, efforts must undergo to define as much as possible new mechanisms involved in the biocontrol of fire blight.

Mass production and **formulation** are two essential steps during the development of a new biological control agent. Both procedures must achieve the goal to assure the maintenance of the antagonistic efficacy after large-scale production of the microorganism, long storage period and during its preparation and inoculation by orchardists. The viability of the produced biocontrol agent is one of the most important

challenges to undertake. Therefore, the formulation and mass production system must attempt to increase the biocontrol agent viability. Moreover, both steps are restricted to an economical threshold to assure the competitiveness of the product in the market (Andrews, 1992; Burgues & Jones, 1998). Several formulations have been developed for the commercialised biocontrol agents of fire blight such as aqueous suspension formulations (Sonata®, Serenade®, Biopro®), concentrated solutions (Serenade®, BS-F4®) and wettable powders (BlightBan A506® , PomaVita®, Blossom Bless®).

Finally, the development of reliable **monitoring methods** that assure the unequivocal identification and quantification of the biological control agent after field release is needed. These methods will provide information about the population dynamics of the biocontrol strain in the target organ. Such knowledge is essential in fire blight management, since the efficacy of the biological control depends on the proportion of colonised blossoms and the population size of the biocontrol agent, which must approach the carrying capacity (Johnson & Stockwell, 1998). The establishment of a population of antagonistic bacteria is influenced by several factors which must be taken into account, such as the method of inoculum preparation, the method of application, the bloom stage and the weather conditions at the moment of application (Johnson & Stockwell, 1998). In addition, the resident indigenous microbiota may influence the capacity of the biocontrol agent to colonise the microhabitat, so studies concerning the ecology of epiphytes on stigmatic surfaces by means of monitoring methods are needed (Johnson & Stockwell, 2000).

MONITORING OF BIOLOGICAL CONTROL AGENTS

A great number of monitoring methods have been developed for the detection and quantification of microorganisms. The level of specificity required, e.g. family, subfamily, genera, species or strain, determines the choice of the method. In the case of biological control agents, a strain-specific detection level is necessary because they are released into the environment, where there is a complex microbial community and other strains belonging to the same species of the biocontrol agent are usually present.

Despite the large variety of monitoring methods described in the literature for the study of microorganisms in nature (Jansson & Prosser, 1997; Van Elsas *et al*., 1998; Olive & Bean, 1999; Schena *et al*., 2000; Björklöf & Jorgensen, 2001; Morris *et al*., 2002), we will focus on those that have been mainly used to monitor biological control agents. Monitoring methods can be divided into three main groups: microbiological methods, direct methods and molecular methods. In all cases, we can differentiate the **detection method**, based on the unambiguous identification of the biocontrol agent by the

selection of a specific marker, from the **quantification method**, which allows to the assessment of its population size. Table 2 summarises several of the monitoring methods described in the literature to track biological control agents.

Table 2: Monitoring methods used to detect and quantify biological control agents

Species	Strain	Detection	Quantification	Reference
Bacteria				
P. agglomerans	C9-1	Ab-R	CFU-count	Nuclo *et al*., 1998
P. agglomerans	EPS125	Ab-R	CFU-count	Bonaterra *et al*., 2005
P. agglomerans	Eh24	Ab-R	CFU-count	Ozaktan & Bora, 2004
Pseudomonas corrugata	2140	Ab-R, *lac*ZY gene rep-PCR	CFU-count	Choi *et al*., 2003
P. fluorescens	A506	Ab-R, *gfp* gene	CFU-count, FCM, Microscopy, Fluor.	Nuclo *et al*., 1998; Lowder *et al*., 2000; Lindow & Suslow, 2003
P. fluorescens	EPS62e	Ab-R	CFU-count	Cabrefiga, 2004
P. fluorescens	EPS288	Ab-R	CFU-count	Montesinos & Bonaterra, 1996
P. fluorescens	SBW25	*gfp*, *lux* genes	CFU-count, FCM, Lum.	Unge *et al*., 1999
P. fluorescens	*phl*D$^+$	Ab-R, rep-PCR	CFU-count, Hybr., MPN-PCR	Landa *et al*., 2002
P. fluorescens	29A	Ab-R, RAPD *gfpmut*2 gene, SCAR	CFU-count Q-PCR	Chapon *et al*., 2002; Chapon *et al*., 2003
P. fluorescens	CHAO	*phl*A gene	CFU-count, QC-PCR	Rezzonico *et al*., 2003
Yeasts				
Aureobasidium pullulans	LS30	Morph., AFLP	CFU-count	Lima *et al*., 2003
A. pullulans	L47	RAPD, SCAR	CFU-count, Real-time PCR	Schena *et al*., 2002
Candida oleophila	I-182	*gus* gene	CFU-count-PCR	Chand-Goyal *et al*., 1998
C. oleophila	O	SCAR	Real-time PCR	Massart *et al*., 2005
Cryptococcus laurentii	LS28	Morph., AFLP	CFU-count	Lima *et al*., 2003
Pichia anomala	K	Morph., SCAR	CFU-count-PCR, QC-PCR-ELOSA	De Clercq *et al*., 2003; Pujol *et al*., 2004
Rhodotorula glutinis	LS11	Morph., AFLP	CFU-count	Lima *et al*., 2003
Fungi				
Hirsutella rhossiliensis	OWVT-1	ITS sequence	Real-time PCR	Zhang *et al*., 2006
Paecilomyces lilacinus	RESP11, 251	ITS sequence	CFU-count, Real-time PCR	Atkins *et al*., 2005
Plectosphaerella cuccumerina	380408	ITS sequence	CFU-count, Real-time PCR	Atkins *et al*., 2003
Trichoderma hamatum	382	RAPD, SCAR	CFU-count-PCR	Abbasi *et al*., 1999
T. harzianum	2413	SCAR	Real-time PCR	Rubio *et al*., 2005
Verticillium chamydosporium	10	β-tubulin gene	CFU-count, C-PCR	Mauchline *et al*., 2002

Ab-R: antibiotic resistance; FCM: Flow cytometry; Fluor.: fluorimetry; Lum.: luminometry; Hybr.: colony hybridisation; Morph.: morphology

Microbiological monitoring methods

These methods, also called classical or culture-based methods, have been commonly used to monitor biological control agents. The main requirement for the culture-based methods is the availability of a selective medium in which only target strain is able to grow or where the target strain is easily differentiable from other strains.

The **morphology** of the colonies of the antagonistic strain has been used as a monitoring tool when there is not a selective medium available. Nevertheless, it can lead to a misleading identification when nontarget strains belonging to the same species are present in the sample. Under this situation, the morphology will not be enough to obtain a reliable detection of the biocontrol agent, and must be used coupled to other markers (Lima *et al*., 2003).

To overcome this problem, several authors have used spontaneous mutants of the target strain showing **antibiotic-resistance** phenotype. The detection of the target strain is then obtained by the growth on a medium supplemented with the antibiotic, where nontarget strains are unable to grow. In this case, it is essential to perform a preliminary study of the microbiota of the plant host to assure that there is not a natural occurrence of the phenotype in the indigenous microbial population. For monitoring fire blight biocontrol agents, two antibiotics have been commonly used as selective markers: rifampicin and nalidixic acid (Nuclo *et al*., 1998; Lindow & Suslow, 2003; Cabrefiga, 2004). When the desired antibiotic-resistance is not achieved in a spontaneous mutation way, the marker can be introduced by genetic transformation of the target strain. In both situations, the antibiotic-resistant strain must express the same characteristics as the parental strain, concerning its fitness and its biocontrol efficacy.

Once the unambiguous detection of the biocontrol agent is assured by one of the methods described above, a method of quantification must be developed and coupled to study its population dynamics after field release.

The population size can be estimated by **colony forming units (CFU) counting** after dilution plating on selective agar media. This method has been widely used for monitoring biocontrol agents because of its easy performance and low cost. In biological control of fire blight, the CFU-counting has been coupled to resistance-antibiotic markers (Nuclo *et al*., 1998; Lindow & Suslow, 2003; Cabrefiga, 2004).

Another method to estimate the population size is the **most probable number** (MPN) determination (Russek & Colwell, 1983). This method is based on the performance of serial dilutions of the sample till the number of viable cells is reduced to zero. Then, each dilution is added and incubated in a selective broth media. The results

are analysed by means of a table, based on the theory of Poisson distribution of small numbers, which provides the probabilistic quantity of microorganisms in the sample depending on the presence or absence of growth at each dilution. The morphology of bacteria is not visible, so the strain must be preferably selected by an antibiotic-resistance marker.

Both CFU-counting and MPN determination are based on the cultivability of the target strain in selective media. The need for cultivability may be a problem due to the selective nature of the growth media that often also inhibits partially the target strain. Therefore, there is another group of culture-independent monitoring methods that have also been developed, called direct monitoring methods.

Direct monitoring methods

Direct monitoring methods are based on the detection of a specific phenotypic characteristic of the biological control agent, such as the emission of fluorescence, to achieve its identification without the need of cultivation. These culture-independent methods offer an alternative measure of the viability of the biocontrol agent population compared to culture-based methods, since bacteria subjected to adverse environmental conditions can lose the cultivability while remaining metabolically active (Kogure *et al*., 1979; Xu *et al*., 1982).

The **bioluminescence** is a phenotypic characteristic that can be used to mark biological control agents. This technique is based on the introduction of an exogenous reporter gene which encodes for enzymes or proteins responsible of bioluminescence. The most frequently described reporter genes have been the *lux* gene from the bacterium *Vibrio fischeri* and the *gfp* gene from the jellyfish *Aequorea victoria*. The *lux* gene encodes luciferase enzymes, and the *gfp* gene encodes green fluorescent proteins. The *lux* and *gfp* genes have been used alone or together. The *gfp* gene was used alone to monitor the biocontrol agent of fire blight *P. fluorescens* A506 (Tombolini *et al*., 1997). The combined use of *gfp* and *lux* genes was chosen to monitor the plant growth promoting bacterium *P. fluorescens* SBW25 (Unge *et al*., 1999) and the biological control agent *Trichoderma harzianum* ThzID1 (Bae & Knudsen, 2000). The transformed strains usually encode for antibiotic-resistance too.

The main advantage of bioluminescence is that the biocontrol agent can be detected *in situ* by non-destructive means. Nevertheless, the main drawback of using bioluminescence, as when using an introduced antibiotic resistance gene, is the need to introduce an exogenous genetic marker to achieve the detection. Therefore, the

biocontrol agent becomes a genetically modified microorganism (GMM), and the environmental release of GMMs is restricted in many countries because of the lack of knowledge of the potential hazards that might be associated with their large-scale use (Van Elsas *et al*., 1998). In Europe, the release of GMMs into the environment is regulated by Council Directive 2001/18/EC.

The quantification in direct monitoring is achieved by optical detection methods such as **fluorescence microscopy** (epifluorescent and laser confocal microscopy), **spectrofluorimetry** or **flow cytometry.** These methods allow the quantification by direct counting of fluorescent bacteria with the microscope, indirect estimation of bacteria by the quantity of fluorescence emitted (spectrofluorimetry), or by counting bacteria according to their morphology, size and range of wavelength emitted (flow cytometry). Microscopy-based methods are tedious since visual counting is required. In contrast, spectrofluorimetry and flow cytometry are automated and the population size is easily obtained.

Some examples of the use of direct monitoring methods for biological control agents in environmental samples are the use of *gfp* marker with flow cytometry to monitor *P. fluorescens* A506::*gfp*2 (Lowder *et al*., 2000), and the use of *gfp*/*lux* dual marker with epifluorescent microscopy, flow cytometry and spectrofluorometry to monitor *P. fluorescens* SWB25::*gfp*/*lux* (Unge *et al*., 1999).

Molecular monitoring methods

There are many molecular methods targeted to nucleic acids. This revision is going to be focused only on those molecular methods based on the detection of nucleic acids by means of polymerase chain reaction (PCR) technique. Furthermore, we want to put emphasis in those that have been used for monitoring biological control agents, besides the fact that there is not too much literature on this matter. Fortunately, molecular monitoring methods are increasingly used in biological control, and the advances on the detection and quantification of pathogens are being applied for monitoring biological control agents.

Concerning the use of molecular markers, the biocontrol agent *P. fluorescens* EPS62e was characterised in a previous work with a specific pattern of macrorestriction fragment length polymorphisms (MRFLP) of genomic DNA followed by pulsed field gel electrophoresis (PFGE) (Cabrefiga, 2004). This method is PCR independent, and consisted of a complete digestion of the genomic DNA with restriction endonucleases. Then, the digested fragments are separated by PFGE to obtain a specific profile for the

target strain (Cirvilleri *et al*., 1996). The profile obtained for EPS62e using the endonuclesase *Swa* I was compared to other *P. fluorescen*s, and the specificity was corroborated. Nevertheless, this method is laborious, time consuming since almost three days are needed to obtain a result, and requires the isolation of the target strain in a pure culture prior to analyse. Therefore, PCR-based methods can simplify this monitoring method.

The development of PCR-based molecular markers to monitor biological control agents is based on the detection of a region within the target strain genome that differentiates it from other strains. For further registration and commercialisation of the biocontrol agent, it is preferable to detect genomic markers instead of introduce an exogenous gene, because the latter will convert the biocontrol agent into a GMM.

When there are known mechanisms involved in the biological control, e.g. antibiosis, the molecular markers can be designed within the target gene. In this way, Raaijmakers *et al* (2001) used the sequence of the gene encoding for the antibiotic 2,4-diacetylphloroglucinol to design PCR-markers to track *P. fluorescens* populations that control root and seedling diseases, and Garbeva *et al.* (2004) used the sequence of the gene encoding for the antibiotic pirrolnitrin to track the population of biocontrol bacteria that suppress a range of phytopathogenic fungi in soil. Besides the use of biosynthetic genes, other characteristics of the biological control agent have been used to design PCR markers, such as the spore coat protein of *B. subtilis* DB170 (Broggini *et al*., 2005). The main drawback of this kind of strategy to obtain PCR-markers is that the detection is not achieved at strain level since the target genes can be present in other nontarget microorganisms.

Despite the use of known sequences, there are useful techniques that provide the detection of natural polymorphisms within the genome of the target strain to obtain strain-specific molecular markers. Some of the most cited techniques in the literature are the amplified fragment length polymorphism (AFLP), the amplification of repetitive-sequences based-PCR (rep-PCR), and the random amplified polymorphic DNA (RAPD). All of these techniques are suitable for many biological control agents since they do not require any prior knowledge of the target strain's genome (Figure 4).

AFLP technique is based on the detection of genomic restriction fragments by PCR amplification (Vos *et al*., 1995). First of all, the genome of the target strain is cut by restriction enzymes and double-stranded adapters are ligated to the ends of the fragments. Then, PCR amplification is conducted by using primers that bind partially to the adapters and partially to the sequence of the adjacent restriction fragment. Therefore, only those fragments in which the sequence matches the primers used will be amplified.

The result is a pattern of amplified products with a high discrimination level between organisms. This technique has been mainly used for eukaryotic biological control agents such as *Rhodotorula glutinis* LS11, *Cryptococcus laurentii* LS28, *Aureobasidium pullulans* LS30 (Lima *et al*., 2003; De Curtis *et al*., 2004) and *Trichoderma* spp. (Buhariwalla *et al*., 2005).

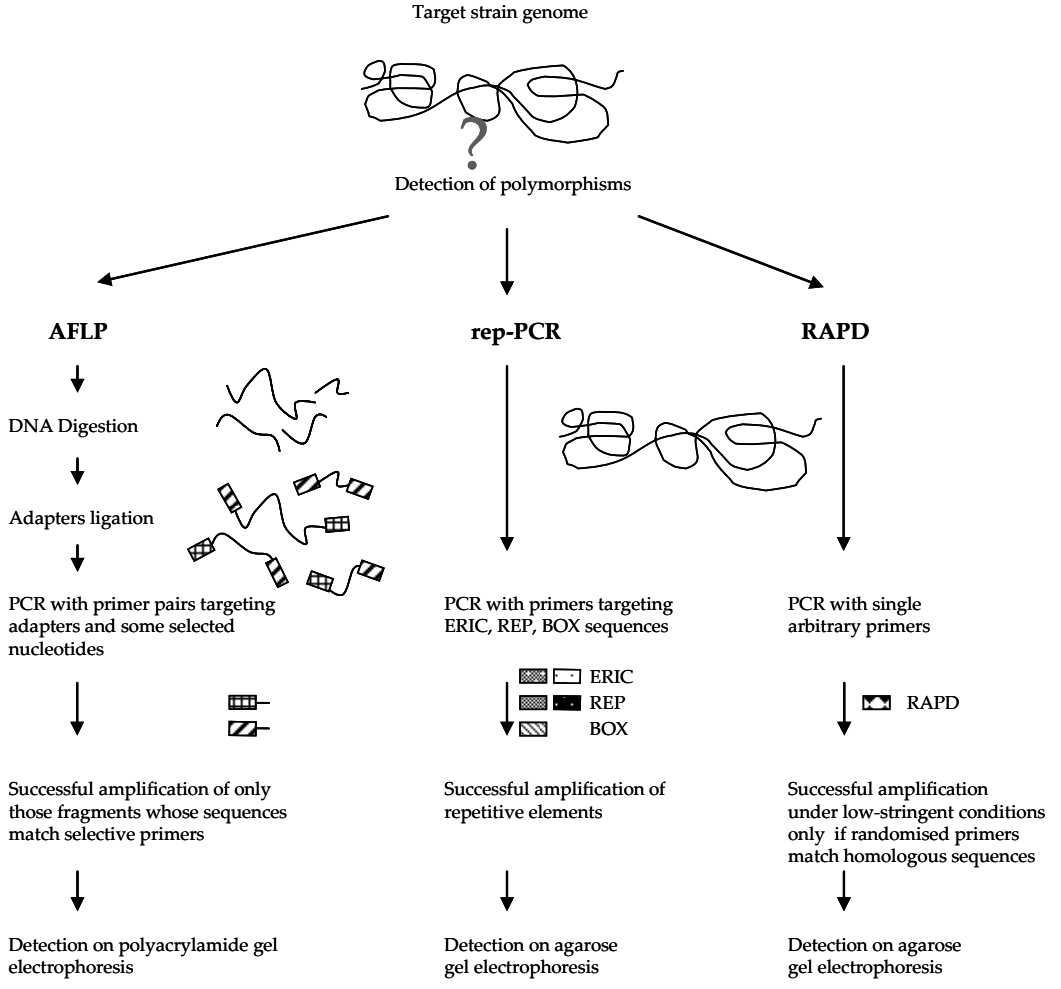

Figure 4: Description of three molecular methods used for fingerprinting biological control agents. AFLP: amplified fragment length polymorphism, rep-PCR: repetitive sequences based-PCR, and RAPD: random amplified polymorphic DNA.

The **rep-PCR** uses bacterial repetitive-sequences such as *enterobacterial repetitive intergenic consesnus* (ERIC), *repetitive extragenic palindromic* (REP), and *box* sequences for primer binding (Versalovic *et al*., 1991). After the amplification, the comparison of the pattern of the amplified products obtained might allow the discrimination among strains. This method is easier to perform compared to the AFLP technique, and it has been used for several bacterial antagonists such as *Pseudomonas corrugata* 2140 (Choi *et al*., 2003), *P. fluorescens* (Landa *et al*., 2002) and *Pseudomonas* spp. (McSpadden Gardener *et al*., 2000).

The **RAPD** technique is based on the use of a single primer for the amplification of genomic DNA within nonrestrictive conditions, i.e. low hybridisation temperature (Welsh & McClelland, 1990; Williams *et al*., 1990). As described for the other two techniques, an amplification pattern is obtained too. The simplicity of this method is similar to rep-PCR; however, RAPD uses randomly-sequenced primers which must be tested empirically in order to find those that bind satisfactorily the target strain, whereas rep-PCR uses known-sequenced primers. The main drawback of RAPD is that the use of unspecific RAPD primers and the low hybridisation temperature can lead to a loss of reproducibility. Nevertheless, the vast majority of works concerning the detection of polymorphisms in biological control agents have used the RAPD technique (Arisan-Atac *et al*., 1995; Raaijmakers & Weller, 2001; Chapon *et al*., 2002; Schena *et al*., 2003).

The methods described above, AFLP, rep-PCR and RAPD, have been traditionally used for fingerprinting microorganisms, but when used for the detection of biological control agents they have an important drawback, because in spite of being specific for the characterisation of the microorganism, they require the isolation of the target strain prior to its detection.

In order to simplify the detection of biological control agents and to overcome the isolation procedure, the methods described above can be performed by developing sequence characterised amplified regions (SCAR) (Figure 5). **SCAR** markers are obtained by the selection of a unique amplified fragment which differentiates the target strain from others. Then, the fragment is sequenced for the design of a primer pair which must allow the specific amplification of a single fragment when working with the target strain. The specificity of the SCAR primers designed must be verified against a large number of strains and species. The SCAR marker simplifies the detection of the antagonist strain because it provides a unique amplification product instead of the amplification pattern obtained by AFLP, rep-PCR or RAPD (Paran & Michelmore, 1993). Furthermore, since the PCR is carried out by specific primers, the conditions of the amplification are stringent, thus the reproducibility of the method is increased. SCAR markers have been

developed for several biological control agents such as bacteria (Chapon *et al.*, 2003), yeasts (Schena *et al.*, 2002; De Clercq *et al.*, 2003) and fungi (LeClerc-Potvin *et al.*, 1999; Becker *et al.*, 1999; Abbasi *et al.*, 1999; Bulat *et al.*, 2000; Paavanen-Huhtala *et al.*, 2000; Hermosa *et al.*, 2001; Taylor *et al.*, 2003; Dauch *et al.*, 2003).

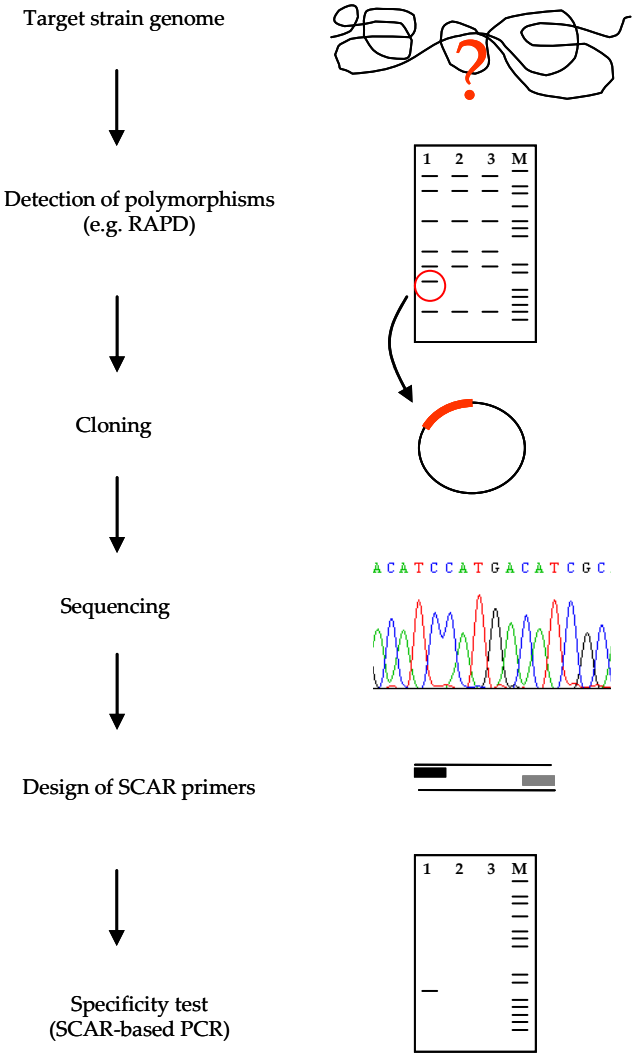

Figure 5: Development of SCAR molecular markers

Since the development of the PCR technique, several techniques have been performed to achieve the quantification as well as the detection of the target DNA. The first quantification method described was the PCR-based dilution end point (Q-PCR)

(Cross, 1995). The **Q-PCR** consisted of serial dilutions of the target DNA prior to amplification, till the concentration is under the detection limit. The initial number of molecules is extrapolated from the dilution factor applied to the last amplifiable sample. The main advantage of the Q-PCR is that it does not require any special equipment for its development. Nevertheless, it is very important the assessment of the reproducibility to obtain a reliable quantitative method. The Q-PCR method was used to monitor the biocontrol agent *P. fluorescens* 29A on the rhizosphere (Chapon *et al*., 2003).

A more complex method of quantification is the quantitative-competitive PCR (QC-PCR) (Zimmermann *et al*., 1996). The **QC-PCR** involves the design of an internal standard DNA, which is co-amplified with the target strain and competes for the primer binding during amplification. Quantification is achieved by comparing the relative yield of the PCR products obtained from the target and the internal standard DNA. The main requirement to obtain a reliable quantification by QC-PCR is that both target and internal standard DNA amplify with the same efficiency. Then, the quantification can be more precise than in Q-PCR method, although QC-PCR requires a much longer development work compared to Q-PCR. This method has been used to monitor the biocontrol agents *Pichia anomala* K (Pujol *et al*., 2004) and *P. fluorescens* CHAO (Rezzonico *et al*., 2003).

More recently, another technique has been increasingly used: the **Real-time PCR** (Higuchi *et al*., 1992). This technique has improved the quantification because data are collected during the reaction, and not only at the end of the reaction at the plateau phase, as happens in Q-PCR and QC-PCR techniques.

The amplification reaction in Real-time PCR is monitored by using fluorescence. First of all, the fluorescent signal used has to be chosen. There are two groups of detection formats in Real-time PCR: the unspecific markers, and the specific probes. The unspecific markers are independent of the target DNA sequence, because they are dyes that become fluorescent when they bound to dsDNA, such as SYBR Green I or AmpliFluor™. The specific probes are oligonucleotides marked with two fluorophors (a reporter and a quencher) homologous to the sequence of the target amplified product, which hybridise with the target DNA during amplification. The fluorescence emitted by the reporter can be absorbed by the quencher depending on the distance (Forster, 1948; Clegg, 1992). If both fluorophors are physically separated at more than 10 nm, the fluorescence is not absorbed by the quencher and can be detected by a charge coupled device (CCD) camera. Specific probes are subdivided in two types: hydrolysis probes (e.g. TaqMan® and TaqMan® MGB) and hybridisation probes (e.g. Molecular Beacons and Scorpion primers). TaqMan® probes are hydrolysed by the 5'-3' exonuclease activity

of the DNA polymerase after each PCR cycle, and this hydrolysis causes an emission of fluorescence proportional to the number of target molecules amplified. The hybridisation probes are not hydrolysed during PCR. In contrast, fluorescence is generated by a change in their secondary structure after binding the target DNA, which is proportional to the number of amplified molecules too.

The quantification of the population size of the biocontrol agent in Real-time PCR is achieved by the development of a *standard curve* and the definition of the *threshold cycle* (C_T) (Figure 6). The standard curve is obtained after the amplification of serial dilutions of known quantities of the target DNA. The fluorescence emitted from each PCR tube is captured during the amplification, and is plotted as a curve (Figure 6A). After the PCR run, the curves of all samples are plotted, and the C_T is defined by choosing the cycle at which fluorescence achieves an established threshold. The C_T values of the standard curve are then plotted against the initial target DNA concentration (Figure 6B). Finally, the initial DNA quantity of an unknown sample is obtained by plotting the C_T of this sample against the standard curve.

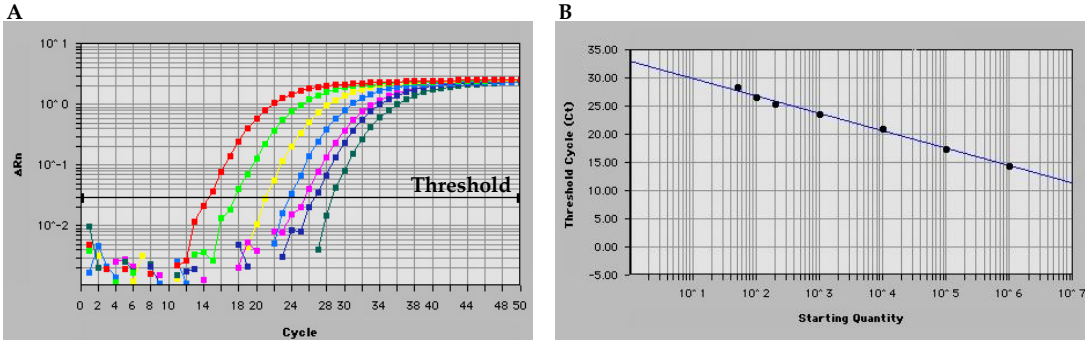

Figure 6: (A) Amplification curves of several dilutions of target DNA (B) Standard curve plotted with the C_T value against the initial quantity of the target DNA.

Since the main objective of developing a Real-time PCR method is the quantification of cells, standard curves departing from viable cells are used as source of DNA. Several strategies can be used to design the standard curve, and the differences are based on the sample used as "standard", also called known sample. One strategy is based on the performance of a titration of purified and quantified DNA, which will be used to extrapolate the number of cells after amplification. A second strategy can be performed by using a titration of purified and quantified DNA mixed in plant extracts.

In this second strategy, the number of cells is also extrapolated from DNA quantity, but the possible presence of PCR inhibitors in the matrix sample is taken into account for quantification. Finally, a third strategy can be performed by using quantified cells, instead of quantified DNA, mixed in plant extracts. In this case, the mixtures follow the DNA extraction procedure and the quantification is not extrapolated from DNA quantity, but from cells quantity. This third strategy involves not only the possible presence of PCR inhibitors in the sample matrix, but also the efficiency of DNA extraction procedure.

Real-time PCR has been used to monitor several biological control agents, mainly fungi (Schena *et al*., 2004) such as *Paecilomyces lilacinus* RESP11 and 251 (Atkins *et al.*, 2005), *Plectosphaerella cuccumerina* 380408 (Atkins *et al*., 2003), *Trichoderma harzianum* 2413 (Rubio *et al*., 2005) and *Hirsutella rhossiliensis* OWVT-1 (Zhang *et al*., 2006), and the yeasts *Aureobasidium pullullans* L47 (Schena *et al*., 2002) and *Candida oleophila* O (Massart *et al*., 2005).

Mixed monitoring methods

This section can not be concluded without thinking that there is not a clearly defined limit between microbiological, direct and molecular methods. Obviously, all methods of detection and quantification described above can be mixed to perform the best monitoring method for each biological control agent. The choice of a monitoring method will depend on the target strain, i.e. the knowledge of its phenotypic and genotypic characteristics, and the advantages and drawbacks of each kind of method.

The advantage of using microbiological culture-dependent methods is that they are easy to perform and require materials and equipment of low cost. In contrast, the main drawback is that only the cultivable population can be detected and quantified, whereas under certain environmental conditions it has been demonstrated that a large part of microbial population can enter into a viable but nonculturable (VBNC) state (Wilson & Lindow, 1992; Ghezzi & Steck, 1999; Mascher *et al*., 2000). Under this situation, the actual population size might be underestimated.

In the case of direct monitoring methods, such as flow cytometry or fluorescence microscopy, the population size is better achieved since all viable cells (cultivable and noncultivable) are counted. The main drawback is that these methods require the use of genetically modified biocontrol agents. Nevertheless, techniques like flow cytometry are very helpful monitoring tools during the development of the biocontrol agent (Tombolini *et al*., 1997; Maraha *et al*., 2004).

Finally, molecular monitoring methods require a long effort for their development and high-cost equipment. In contrast, once developed they are less time-consuming, more sensitive, and culture-independent. The most important drawback of these methods is that the viable population might be overestimated since they do not differentiate between living and dead cells (Schena *et al*., 2004).

In order to overcome the drawbacks of using a single microbiological, direct or molecular method, they can be combined, and there are several examples in the literature concerning the combined use of methods for monitoring biological control agents. Maucline *et al.* (2002) developed a competitive PCR (cPCR) to monitor the biocontrol agent of pathogenic nematodes *Verticillium chlamydosporium*, and combined it with selective plating. In their study the cPCR was less variable than plate counts to estimate the biocontrol population level. Another work performed by Rezzonico *et al.* (2003) developed a QC-PCR method to track the biocontrol agent of soil-born pathogens *P. fluorescens* CHAO, and combined it with CFU-counting method. They showed that the correlation between both methods depended on the physiological state of cells, being positive when cells grew without stressful conditions, and negative under stress. Finally, Atkins *et al.* (2003) and Atkins *et al.* (2005) developed Real-time PCR methods for the nematophagus fungi *Plectosphaerella cucumerina* and *Paecilomyces lilacinus* and compared the molecular methods developed to other traditional methods such as dilution plating. The estimated values between methods differed in some cases, and they concluded that the combined use of them was needed and provided useful data concerning biocontrol efficacy.

A NOVEL BIOCONTROL AGENT OF FIRE BLIGHT, *Pseudomonas fluorescens* EPS62e

The Plant Pathology Group of the University of Girona is developing a novel biological control agent of fire blight (Cabrefiga *et al*., 2002; Cabrefiga *et al*., 2003). In a previous PhD thesis (Cabrefiga, 2004), *P. fluorescens* EPS62e was selected as a potential biocontrol agent of fire blight. This strain was identified, characterised and the putative mechanisms of action were studied. A brief description of the major advances concerning the development of this biological control agent is given below.

P. fluorescens EPS62e was isolated in a screening procedure based on a selective enrichment, in which plant extracts that contained a microbiota mixture were tested in immature pear fruit wounds for their ability to inhibit *E. amylovora* infection. In fact, EPS62e was isolated from an extract of a healthy Conference pear fruit from Riudellots (Girona) that showed high efficiency in the control of fire blight on immature pear fruit. The strain EPS62e was identified as a *P. fluorescens* species by using the API 20NE system

(Biomerieux, France) and other biochemical tests such as oxidative-fermentative metabolism, oxidase test and fatty acids profile. It was shown that EPS62e produced siderophores, but it did not produce cyanide, chitinases, indolacetic acid, and any of the most frequently antibiotics described in *P. fluorescens* species such as 2,4-diacetylphloroglucinol, phenazine-1-carboxylic and pyrrolnitrin in the media tested. The lack of production of these substances explained the absence of *in vitro* inhibition of pathogens like *Xanthomonas campestris*, *E. amylovora* and *P. expansum* by EPS62e. In contrast, EPS62e efficiently inhibited *E. amylovora* infections in immature pear fruits, blossoms and pear potted plants. The selected strain showed an efficacy of 65% in the reduction of severity and of 60% in the reduction of incidence of blossom blight (Figure 7); and reduced 96 % *E. amylovora* incidence and severity of shoot blight when applied at 10^8 CFU/mL. In all the assays performed, the efficacy of disease control depended on the arrival of the biocontrol agent prior to the pathogen colonisation.

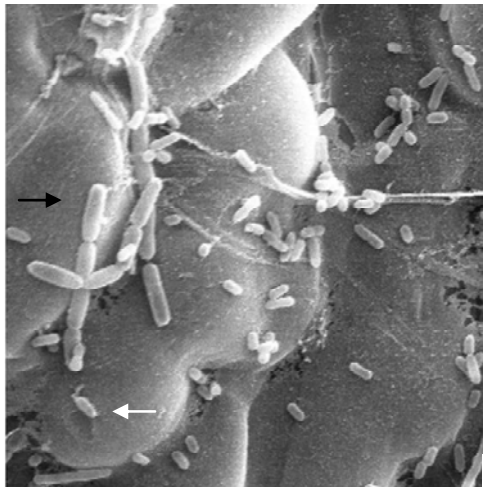

Figure 7: Scanning electron micrographs of cv. Doyenne du Comice pear flowers colonised by *P. fluorescens* EPS62e (black arrow) and *E. amylovora* EPS101 (white arrow) (Cabrefiga, 2004)

The major mechanism of action of EPS62e to control fire blight is based on a preemptive exclusion by nutrient depletion and site colonisation. *E. amylovora* have similar nutritional requirements, thus under limited nutrient environment conditions, EPS62e can compete and exclude *E. amylovora* from the habitat. Furthermore, EPS62e have a higher growth rate than *E. amylovora* on immature pear fruits. Nevertheless, preliminary assays have indicated that cell-to-cell interaction can also contribute to the

biocontrol efficacy of EPS62e, but more studies are required to comprehend this kind of mechanism in *P. fluorescens* EPS62e.

The strain EPS62e is quite tolerant to some of the chemicals used to control fire blight at the recommended commercial concentrations (copper hydroxide, kasugamycin and streptomycin) and sensible to others (copper sulphate, copper oxychloride and fosetyl-aluminium). This biocontrol agent has a wide range of antagonism against *E. amylovora* strains on immature pear fruit assays. For its further commercialisation, EPS62e can be large-scale produced by fermentation, and long term storage has been performed by freeze-drying. Nevertheless, optimisation of its formulation and mass production are being studied.

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OBJECTIVES

The aim of this work was to develop molecular monitoring methods for *P. fluorescens* EPS62e to assess its environmental fate after field release. Therefore, the work consisted of:

1. The analysis of molecular fingerprints to discriminate EPS62e from other *P. fluorescens* strains.
2. The development of PCR based methods for specific monitoring of the strain EPS62e.
3. The study of the environmental fate of *P. fluorescens* EPS62e in apple and pear.

RESULTS

ANALYSIS OF MOLECULAR FINGERPRINTS AND DEVELOPMENT OF PCR BASED METHODS FOR SPECIFIC MONITORING OF *Pseudomonas fluorescens* EPS62e

The registration of a novel biocontrol agent involves the development of monitoring methods that allow its specific detection and differentiation from other strains belonging to the same species and to closely related species. The further purpose of the development of such biopesticide is to release the target strain in orchards, where other microorganisms are naturally present. Therefore, the unambiguous detection of the biocontrol agent in a complex microbiological and plant matrix is necessary.

Preliminary studies of EPS62e colonisation of different plant organs were carried by CFU-counting on selective media of a spontaneous mutant of EPS62e resistant to nalidixic acid which retained the phenotypical characteristics and biocontrol efficacy of

the parental strain. The specific detection of the strain EPS62e was corroborated by MRFLP-PFGE specific profile. Even though this monitoring method was performed in some preliminary colonisation studies, MRFLP-PFGE technique was not suitable for routine monitoring assays of the biocontrol agent because of its complexity and the need to obtain a pure culture of the target strain prior to analyse.

The development of a monitoring method for a biocontrol agent requires specificity at strain level, which is usually achievable by molecular methods. However, in the vast majority of cases the poor knowledge of the biocontrol agent genome makes difficult the finding of such molecular markers. Sometimes, specific markers can be obtained by sequencing a fragment of the target strain genome. There are some regions of the genome that are less conserved than others, such as the internal transcribed spacer (ITS). The comparison of the sequenced region against other sequences from the GenBank database may allow the design of primers if there are enough differences to discriminate among strains. If the sequences are not available, or there are not enough differences to design specific PCR-markers, other fingerprinting methods that do not require previous knowledge of the target genome's sequence can be developed.

Among the large diversity of fingerprinting methods, the RAPD has been commonly used to detect specific amplified patterns of biological control agents. The great advantage of using RAPD method is that it can be easily performed, and if successful, it provide distinctive amplified fragments that can be recovered from gels, cloned and sequenced for the design of a SCAR marker. The SCAR marker is based on a specific primer pair that allows the unambiguous detection of the target strain by conventional PCR amplification.

Conventional PCR-based method will assure the specific detection of the target strain, and it can be easily coupled to CFU-counting or MPN determination on selective media by using a spontaneous antibiotic-resistant mutant to obtain a quantitative method to asses the population level of the biocontrol agent.

The combined use of culture-based methods and molecular methods offers a high level of specificity, since the PCR-method can be coupled to analyse randomly picked colonies or broth media after bacterial growth to corroborate the identification of the target strain.

The aim of this work was, firstly, to sequence the 16S rDNA and the ITS region to characterise EPS62e and to analyse the similarities from this region with sequences of other *P. fluorescens* strains in the GenBank database. Secondly, to detect natural polymorphisms in the genome of EPS62e which discriminate it from other strains by means of RAPD and Unspecific-PCR (U-PCR). Thirdly, to develop SCAR markers to

obtain a rapid and simple PCR-based method to detect EPS62e; and finally, to couple the PCR-based method to two culture-based methods, CFU-counting and MPN determination, to perform a specific-quantitative monitoring method for the biological control agent EPS62e.

The methodology, results, discussion and conclusions are presented below as it was published in the journal of the Federation of European Microbiological Societies, *FEMS Microbiology Letters*.

PAPER 1

"Development of a strain-specific quantitative method for monitoring *Pseudomonas fluorescens* EPS62e, a novel biocontrol agent of fire blight". *FEMS Microbiology letters.* Volume 249, issue 2 (August 2005) : p. 343-352

http://www.blackwell-synergy.com/doi/full/10.1016/j.femsle.2005.06.029

ABSTRACT

Pseudomonas fluorescens EPS62e has been selected in a screening procedure for its high efficacy controlling *Erwinia amylovora* infections in flowers, immature fruits and young pear plants. We developed two monitoring methods which allowed specific detection and quantification of EPS62e by combining classical microbiological techniques with molecular tools. RAPD and unspecific-PCR fingerprints were used to differentiate EPS62e from other *P. fluorescens* strains. Differential amplified fragments from EPS62e were sequence characterized as SCAR markers and two primer pairs were designed and selected for their specificity against EPS62e. A SCAR primer pair was evaluated and validated for the assessment of population dynamics of EPS62e on pear plants under greenhouse conditions using plating and most probable number assays coupled to PCR. Both techniques were useful in monitoring the biological control agent. The population level of EPS62e after treatment was 7 log CFU (g f.w.)$^{-1}$, which in turn decreased progressively to 4–5 log CFU (g f.w.)$^{-1}$ after 17 days and then remained stable until the end of the assay 11 days later. The limit of detection of both monitoring methods developed was around 3 log CFU (g f.w.)$^{-1}$, thus, providing a reliable tool for the analysis of EPS62e in greenhouse or field trials, and the assessment of threshold population levels for efficient biocontrol of fire blight.

PAPER 2

"Assesment of the environmental fate of the biological control agent of fire blight, *Pseudomonas fluorescens* EPS62e, on apple by culture and real-tim PCR methods". *Applied and environmental microbiology.* Volume 72, no. 4 (April 2006) : p. 2421-2427

http://aem.asm.org/cgi/content/full/72/4/2421

ABSTRACT

The colonization of apple blossoms and leaves by *Pseudomonas fluorescens* EPS62e was monitored in greenhouse and field trials using cultivable cell counting and real-time PCR. The real-time PCR provided a specific quantitative method for the detection of strain EPS62e. The detection level was around 10^2 cells g (fresh weight)$^{-1}$ and the standard curve was linear within a 5-log range. EPS62e actively colonized flowers reaching values from 10^7 to 10^8 cells per blossom. In apple flowers, no significant differences were observed between population levels obtained by real-time PCR and plating, suggesting that viable but nonculturable (VBNC) cells and residual nondegraded DNA were not present. In contrast, on apple leaves, where cultivable populations of EPS62e decreased with time, significant differences were observed between real-time PCR and plating. These differences indicate the presence of VBNC cells or nondegraded DNA after cell death. Therefore, the EPS62e population was under optimal conditions during the colonization of flowers but it was stressed and poorly survived on leaves. It was concluded that for monitoring this biological control agent, the combined use of cultivable cell count and real-time PCR is necessary.

PAPER 3

"Epiphytic fitness of a biological control agents of fire blight in apple and pear orchards under Mediterranean weather conditions". *FEMS Microbiology ecolgy.* Volume 59, issue 1 (January 2007) : p. 186

http://www.blackwell-synergy.com/doi/full/10.1111/j.1574-6941.2006.00227.x

ABSTRACT

The behaviour of *Pseudomonas fluorescens* EPS62e was investigated in apple and pear orchards under Mediterranean climatic conditions. The trials studied the influence of weather conditions, plant host species, presence of indigenous microbial community and spread from treated to nontreated trees on colonization and survival. Population dynamics were assessed by real-time PCR and CFU-counting methods. With inoculated flowers, weather conditions were optimal for colonization, and EPS62e established high and stable population levels around 10^8 CFU per organ, according to both methods of analysis. The plant host species did not influence the colonization rate, and the biocontrol agent dominated the microbial communities of blossoms, representing up to 100% of the total cultivable population. With inoculated leaves, the EPS62e population decreased to nondetectable levels 30 days after treatment according to both methods used. EPS62e spread moderately in the orchard, being detected in nontreated flowers of trees 15–35 m from the inoculation site. The combined use of real-time PCR and CFU-counting methods of analysis permitted the identification of three physiological states for EPS62e in the field, which consisted of active colonization, survival and entry into a viable but nonculturable state, and cell death.

GENERAL DISCUSSION

Biological control of fire blight disease of rosaceous plants has been developed and performed during the last 25 years (Johnson & Stockwell, 2000), because it represents a reliable complementary method for an integrated pest management (Fravel *et al*., 1999; Montesinos, 2003; Fravel, 2005). Several biocontrol agents are under commercialisation in the United States, and their implementation have improved the control of fire blight, overcoming the negative effects occurred after years of treatments with chemical products such as the appearance of antibiotic-resistant strains of the pathogen (Moller *et al*., 1981; Loper *et al*., 1991; Jones & Schnabel, 2000). In Europe, the implementation of biological control of fire blight is being developed too, and some strains are under registration according to European Directives 91/414/EEC and 2001/36/EC. In that sense, *P. fluorescens* EPS62e was selected in the Plant Pathology Group of the University of Girona as a potential biocontrol agent of fire blight because it showed high efficacy

controlling the disease in immature pear fruits, flowers and potted pear plants (Cabrefiga, 2004).

The implementation of a biocontrol agent for fire blight management involves different phases, such as the development of a monitoring method that assures its specific detection and quantification after field release. To guarantee the efficacy of the biocontrol agent, it must be able to colonise and survive in the target organ (Van Elsas *et al*., 1998). Therefore, it is essential to know the behaviour of the biocontrol agent after field release prior to registration.

Culture-based monitoring methods have been traditionally developed by means of spontaneous mutants of the target strains with resistance to antibiotics (Johnson *et al*., 2000; Mercier & Lindow, 2001; Bonaterra *et al*., 2003; Cabrefiga, 2004). The main advantages of culture-based methods are that they do not require any special equipment, are easy to develop, and are not expensive to set up. However, these methods are laborious, time consuming, and can underestimate the population level if the biocontrol agent enters in a VBNC state (Van Elsas *et al*., 1998; Knight, 2000). Culture-independent alternative methods have also been developed to overcome these drawbacks, such as those methods based on the PCR (Knight, 2000). The PCR-based molecular monitoring methods have the advantage that are high specific and sensitive compared to culture-based methods, and the spontaneous mutant of the target strain is no longer necessary since they can target specific DNA sequences of the wild type strain. Moreover, the PCR technique can be improved to obtain the quantification of the target strain by means of Real-time PCR development (Higuchi *et al*., 1992). Nevertheless, the drawbacks of PCR-based methods are that they require a long effort for being developed, special equipment, a higher cost compared to culture-based methods, and they can not discriminate between living and dead cells (Schena *et al*., 2004).

The present doctoral thesis aimed to contribute to the success of *P. fluorescens* EPS62e as a biocontrol agent of fire blight by means of the development of a specific Real-time PCR monitoring method, and through the study of its behaviour after field release using Real-time PCR and culture-based methods.

The development of a Real-time PCR required (i) the detection of genomic polymorphisms within EPS62e genome which differentiated the target strain from other strains of the same species; (ii) the selection of differential amplified fragments from the fingerprinting profile for the development of SCAR markers; (iii) the design of a conventional PCR based on the SCAR markers for the specific detection; and (iv) the design of a Real-time PCR for its quantification.

The first approach to obtain a molecular marker to discriminate *P. fluorescens* EPS62e from other strains was based on the sequencing of the 16S rDNA and the ITS genomic regions. The 16S rDNA gave us an identity card for the biocontrol agent, contributing to the requirements for registration. The variable ITS region has been increasingly used for phylogeny and population diversity studies (Boyer *et al*., 2001), and several authors have used these sequences to design specific PCR markers for several biocontrol fungi (Atkins *et al*., 2003; Atkins *et al*., 2005; Zhang *et al*., 2006). Even though the sequences obtained from 16S rDNA and ITS regions were useful for the genotypic characterisation of EPS62e, and were deposited in the GenBank database with the accession number AJ583090, the differences observed within the ITS region, compared with other sequences of *P. fluorescens* strains, were not enough to design a specific PCR marker.

The second approach to detect polymorphisms within EPS62e genome was based on the research of a molecular marker without prior knowledge of the target DNA sequence. RAPD technique was chosen for its potential to obtain specific molecular markers when working with other biocontrol agents (Arisan-Atac *et al*., 1995; Droby *et al*., 1999; Raaijmakers & Weller, 2001; Chapon *et al*., 2002; De Clercq *et al*., 2003). U-PCR was a new methodology performed in this work, based on the use of sequence-defined primer pairs under typical thermal conditions of RAPD amplification. In a previous work concerning the production of antifungal secondary metabolites by *P. fluorescens* strains such as phenazine-1-carboxylic acid, diacetylphloroglucinol, pyrrolnitrinand and pyoluteorin (Badosa, 2001), some strains that did not present the biosynthetic gene analysed by specific PCR showed unspecific amplifications, with patterns similar to RAPD profiles. Since EPS62e did not express any of the biosynthetic genes, the results encouraged the usefulness of those primers in a U-PCR.

RAPD and U-PCR methods gave rise to amplified fragment patterns that discriminated EPS62e from other strains. The high heterogeneity among strains belonging to *P. fluorescens* species (Palleroni & Moore, 2004) had likely favoured the finding of specific amplified fragments in EPS62e RAPD and U-PCR profiles.

The use of a RAPD or U-PCR profiles to detect a biological control agent requires the isolation of the target strain prior to its detection, and are tedious methods that can be simplified by the development of SCAR markers, based on the specific amplification of a single PCR fragment (Paran & Michelmore, 1993). We selected three amplified fragments from RAPD and U-PCR profiles that were sequence characterised as SCAR markers. Primers were designed on each SCAR sequence and were screened against 161 strains belonging to the same species, 75 strains from closely related species and 61 field

samples to evaluate their specificity and suitability to become specific molecular markers for EPS62e detection. Among the three SCAR primer pairs designed, one of them was rejected because it showed unspecific amplifications, and the two others were adequate and therefore retained. The lack of specificity of some of the SCAR markers obtained from RAPD profiles had also been described in the literature for the biocontrol fungus *Beauveria bassiana* (Castrillo *et al*., 2003), so to increase the probability to find a strain-specific SCAR marker is recommended the selection of more than one RAPD or U-PCR fragment, whenever possible.

Once conventional PCR was successfully developed for the specific detection of the biocontrol agent EPS62e, we pursued with the design of the quantitative method. For this purpose, both specific SCAR sequences were used to design a Real-time PCR for each with TaqMan® probes and primer pairs targeting shorter internal sequences. The development of Real-time PCR involved the optimisation of the reaction, the verification of the specificity and sensitivity, and the evaluation of the accuracy of the quantitative method, i.e. the standard curve. Fortunately, the two optimised Real-time PCR designs were specific for the detection of EPS62e, with a limit of detection situated at 10^2 CFU g fw^{-1}. Three standard curves were developed and evaluated to establish the best procedure for further assays. Even though the three strategies showed good linearity (R^2 > 0.99), the quantification method chosen was based on a standard curve of several dilutions of EPS62e cells mixed with plant extracts prior to DNA extraction. This standard curve offered the advantage that it took into account the efficiency of DNA extraction procedure, and the presence of PCR inhibitors in the sample (Zeng *et al*., 2004).

The Real-time PCR monitoring method was then used and compared with a culture-based method to track EPS62e population levels. Both methods should contribute to comprehend the behaviour of the biocontrol agent in different plant host tissues, species, and weather conditions.

First of all, an assay was performed to compare and choose a culture-based method to be used later with Real-time PCR for the validation assays. A conventional PCR based on SCAR markers was coupled to two microbiological quantitative methods, CFU-counting and MPN determination, to monitor EPS62e population on pear leaves. The assay was conducted on potted pear plants sprayed with the biocontrol agent under controlled environment conditions. Samples were collected periodically and were analysed simultaneously by CFU-counting-PCR and MPN-PCR. Both CFU-counting-PCR and MPN-PCR were useful to track EPS62e population dynamics on pear leaves, and provided us with useful data to better understand the behaviour of the biocontrol agent in pear leaves. Nevertheless, the CFU-counting method was retained, because it

has been the most common method used to track biocontrol agents of fire blight in field studies (Johnson *et al*., 1993; Lindow *et al*., 1996; Stockwell *et al*., 1996; Nuclo *et al*., 1998; Lindow & Suslow, 2003).

Real-time PCR and CFU-counting methods were used simultaneously in a first approach to evaluate their reliability in the monitoring of EPS62e in apple blossoms and leaves under greenhouse and field conditions. The results showed that both monitoring methods correlated well in the assays performed with flowers, whereas they showed a lower correlation when working with leaves. It was concluded that, under favourable conditions, such as the colonisation of flowers, both methods were useful to monitor EPS62e. However, under nonfavourable or stressful conditions such as the colonisation of leaves, the presence of VBNC cells or nondegraded DNA after cell death caused a discrepancy between data estimated by Real-time PCR and CFU-counting methods, and so both methods of monitoring were required.

The last part of the doctoral thesis, once the methodology was developed and optimised, aimed to evaluate the epiphytic fitness of EPS62e in field after its inoculation on two economical important species affected by *E. amylovora*, pear and apple trees. During this study, the influence of climatic conditions on its colonisation efficiency was evaluated, as well as the influence of EPS62e introduction on the indigenous microbiota and its ability to spread from inoculated to noninoculated blossoms in the orchard.

We did not observe any remarkable difference between the colonisation pattern of EPS62e on pear and apple blossoms. Both species were optimal for the growth of EPS62e, and population levels reached the carrying capacity of blossoms, from 10^7 to 10^8 CFU/blossom. These results are in agreement with other reports on fire blight biocontrol agents such as *P. fluorescens* A506 which attained population levels of 10^6 to 10^7 CFU/stigma (Johnson & Stockwell, 1998; Lindow & Suslow, 2003), *P. agglomerans* C9-1 which population on blossoms averaged 10^4 to 10^6 CFU/blossom (Johnson *et al*., 1993; Nuclo *et al*., 1998; Stockwell *et al*., 1998) and *P. agglomerans* Eh252 which reached mean population size from 10^6 to 10^7 CFU/blossom (Johnson *et al*., 2000). Therefore, our results indicated the high efficiency of the biocontrol agent EPS62e to colonise flowers, the main pathway of *E. amylovora* infections (Vanneste & Eden-Green, 2000). The establishment of a population of antagonistic bacteria in blossoms has been described as the most critical step to implement biocontrol of fire blight in commercial orchards (Johnson & Stockwell, 1998). The stage of bloom must be considered when applying biocontrol agents. In the present work we demonstrated that a single application during bloom was enough to reach high colonisation rate of the biocontrol agent EPS62e. However, this kind of application involves the risk of prior establishment of *E. amylovora* during early bloom;

therefore two applications are recommended at early and later bloom stages to overcome this problem.

Field experiments, where the biocontrol agent was inoculated on apple leaves, confirmed that EPS62e was not an efficient colonising bacterium of the phyllosphere, unless it could be formulated to be better adapted to this habitat, described in the literature as a harsh environment for bacterial colonisation (Lindow & Brandl, 2003; Bailey, 2004).

The overall analysis of the results obtained from field experiments in France and Spain during spring-summer 2004 and 2005 by using simultaneously Real-time PCR and CFU-counting, permitted the identification of three situations related to the fitness of EPS62e. Firstly, when the population level gave similar and increasing values with both methods of analysis, EPS62e was under active colonisation without obvious stress. Secondly, when the population levels decreased but stabilised with time with higher values of Real-time PCR than CFU-counting, EPS62e was under suboptimal colonisation conditions and likely entered into a VBNC state. Thirdly, when the population levels decreased with time in both methods, EPS62e was under unfavourable conditions and cells dead.

Weather conditions were monitored during the field experiments to interpret possible changes in biocontrol agent colonisation, as has been observed for other fire blight biocontrol agents (Nuclo *et al*., 1998; Johnson *et al*., 2000; Pusey, 2002; Lindow & Suslow, 2003; Thomson & Gouk, 2003; Pusey & Curry, 2004). The parameters measured were temperature, relative humidity and rainfall. The population levels of EPS62e on blossoms were stable during the assays performed in the spring 2004 in France and the spring 2005 in Spain. These results did not bring out direct influences of weather in EPS62e population. Nevertheless, Johnson *et al.* (2000) have shown that there is a positive correlation of mean daily temperatures and a negative correlation of rainfall periods with the population size of the biocontrol agent *P. agglomerans* C9-1S in pear and apple blossoms. In the US, Stockwell *et al.* (2002) and Johnson *et al*. (2004) developed a weather-forecast-based predictive model to optimise the timing of applications of bacterial antagonists. To conclude about the influence of weather in EPS62e population and improve its timing of application, further field studies in the same region during consecutive springs should be performed.

The influence of the introduced EPS62e strain on indigenous microbiota was evaluated by comparing the total cultivable population levels with the cultivable EPS62e population. It was observed that when EPS62e was under optimal conditions in pear and apple blossoms, it was able to dominate the bacterial habitat, representing 100 % of the

total bacteria recovered. These results are in agreement with other reports concerning *P. fluorescens* A506 (Wilson & Lindow, 1993) and *P. agglomerans* C9-1 (Johnson *et al*., 2000), in which it was concluded that both biocontrol agents appeared to be relatively resilient to competition from indigenous microbiota, since their growth was not affected by the presence of the resident bacterial population. The ability of EPS62e to compete and dominate the microbiota of flowers is in accordance to its main mechanism of action, based on a preemptive competitive exclusion (Cabrefiga, 2004). In contrast, when the environment was not optimal, such as on apple leaves, EPS62e population levels decreased and it could not compete with the indigenous microbiota for the ecological niche. One possible explanation is that the low nutrient availability of leaf surface compared to flower stigmata caused that EPS62e competed without successful for identical limited resources with the indigenous microbiota (Kinkel & Lindow, 1993). Moreover, in an environment such as leaf surface, where there is a high degree of resource aggregation (Kinkel *et al*., 2002), the immigration and growth of EPS62e was limited. In the spatial model for microbial colonisation of leaves proposed by Kinkel *et al.* (2002), the local density can directly influence microbial growth or survival rate. Consequently, it is likely that the presence of resident competitors directly reduced EPS62e growth and survival rate by reducing the availability of nutrients.

One of the advantages of setting up a biocontrol strategy for fire blight management is the capacity of certain antagonists to spread within trees. Several apple and pear trees have asynchronous flowering, which represent a high risk since new opened flowers that were not treated with the biocontrol agent risked to be colonised by the pathogen. In this respect, we studied the dispersal capacity of EPS62e by insects, rain and wind, as a likely added value for its commercialisation. The dispersal assay showed that the biocontrol agent EPS62e was able to move from treated to nontreated blossoms in the orchard, reaching high population levels, as described for the commercialised biocontrol agent of fire blight *P. fluorescens* A506 (Lindow & Suslow, 2003). These results encourage the further development of EPS62e as a biopesticide in the EU market.

In summary, the development of Real-time PCR as a monitoring method for the biocontrol agent EPS62e, combined with culture-based methods, provided us with useful tools to study the behaviour of this strain after field release. The environmental fate of the strain has been analysed under greenhouse and field conditions, on apple and pear trees, and at present, several questions have been worked out. Nevertheless, it remains some question marks to solve, such as the demonstration of the entry of EPS62e cells in a VBNC state, and the study of the degradation rate of DNA after cell death. On this matter, a new monitoring method is being developed, based on the quantification of a *P. fluorescens* EPS62e::gfp mutant by using flow cytometry (FCM). Despite the fact that the

mutant should not be suitable for commercialisation, it could be very useful during the development of the biopesticide to understand the differences observed in EPS62e population between culture-based method and Real-time PCR under unfavourable environments. In fact, the FCM quantification method assures the detection of living cells, e.g. by using propidium iodide to stain cells with damaged/leaked membranes (Gruden *et al*., 2004). These staining procedure coupled the fluorescence emitted by *gfp* tagged cells, will allow the detection of total living EPS62e cells, as has been described for other biocontrol agents (Tombolini *et al*., 1997; Maraha *et al*., 2004). The development and validation of FCM for the monitoring of EPS62e::*gfp*, and its combined use with culture-based methods and Real-time PCR will provide the knowledge of the contribution of VBNC cells and nondegraded DNA to a more detailed study of the fitness of *P. fluorescens* EPS62e after its release in an unfavourable environment.

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CONCLUSIONS

1. A specific amplification pattern was obtained by PCR-based methods to differentiate EPS62e from other strains belonging to the same species. The strain was characterised by sequencing the 16S rDNA and ITS regions, and polymorphisms within its genome were found by means of RAPD and U-PCR methods.
2. Three discriminatory fragments from RAPD and U-PCR profiles were converted into SCAR markers, but only two of them showed specificity for the detection of EPS62e by conventional PCR.
3. Two culture-based methods coupled to PCR, CFU-counting-PCR and MPN-PCR, were useful for the monitoring of the cultivable population of EPS62e.
4. The Real-time PCR designed on the basis of SCAR markers was fully specific and highly sensitive for the detection and quantification of the biocontrol agent

EPS62e. The quantification was optimised with a standard curve based on mixing EPS62e cells with plant extracts prior to DNA extraction, and it showed linearity over a 5-log range.

5. The Real-time PCR was validated in comparison with CFU-counting method for monitoring EPS62e, and the results provided useful information about EPS62e fitness in controlled environment and field experiments.

6. No significant differences were observed between Real-time PCR and CFU-counting methods when EPS62e colonised apple and pear blossoms in the field, which indicated that VBNC cells and nondegraded DNA were not present or at least at detectable levels. Springtime weather conditions from the northwestern of France in 2004 and the northeastern of Spain in 2005 were suitable for EPS62e blossom colonisation, and under those conditions the strain colonised flowers without obvious stress.

7. The biocontrol agent EPS62e colonised blossoms till high population levels, from 10^7 to 10^8 CFU/blossom. This population was maintained six weeks after treatment during fruit formation, where EPS62e was distributed mainly in the calyx area. Moreover, it dominated the total microbiota of flowers, representing 100 % of the cultivable population few days after treatment.

8. Significant differences were observed between Real-time PCR and CFU-counting methods when EPS62e was inoculated in apple leaves in greenhouse and field. In general, population levels obtained by Real-time PCR were higher than those obtained by CFU-counting method on leaves. Under these conditions, EPS62e was supposed to be stressed because of the harsh environment of the phyllosphere, so cells could enter into a VBNC state being nondetectable by CFU-counting or dead.

9. One single treatment of EPS62e at late bloom stage was enough to reach the high population levels, near the carrying capacity of flowers, that has been described as necessary to prevent *E. amylovora* infections.

10. The strain EPS62e exhibited a moderate spread, being able to colonise noninoculated trees situated 15 to 35 m far from inoculated sites. This capacity represented an advantage compared to chemical control, thus permitting redistribution of the biopesticide during asynchronous flowering occurring in most apple and pear trees.

AGRAÏMENTS

Aquesta tesi és el fruit d'un llarg treball que no hauria estat possible sense la participació de moltes persones a les que vull donar les gràcies.

En primer lloc, als meus directors, l'Emili Montesinos i l'Esther Badosa. Gràcies per ajudar-me a dur a terme aquesta tesi. He après molt amb vosaltres i ha estat un plaer treballar plegats. Gràcies pels consells, les crítiques, les felicitacions i la vostra confiança.

També vull agrair a tots els companys de l'àrea de patologia vegetal que han contribuït a crear el bon ambient de treball. A la Lídia, la causant que jo aparegués per Girona tornant de Bèlgica, gràcies. A en Jordi i en Jesús, els meus primers companys de despatx i de molts *karaokes* després. A l'Anna, la Cun, l'Isidre i en Pere, que sempre han tingut un moment per donar-me un consell. A la Mary, perquè plegades no només hem compartit la realització d'una tesi sinó també una gran amistat. A l'Olga, la Carme, la Roser, l'Albert, la Lourdes, la Rosalia, la Patrícia, l'Anna, en Ferran, la Gemma, en Josep... gràcies a tots.

A tots els companys de l'INRA d'Angers, que van fer de la meva estada una molt bona experiència científica i personal. A en Charles Manceau i el seu equip, per acollir-me com un membre més. A en Jean-Pierre Paulin i l'equip de foc bacterià, que van fer possible tots els assajos d'hivernacle i camp. A totes les persones que vaig conèixer al centre, i molt especialment a en Pierre, l'Arnaud, en Fabien i l'Emilie.

A la Mary Jane, en Troy, la Roser i en Sebastien, per les correccions lingüístiques.

A en Vicenç, del servei de Microscopia de la UdG, per facilitar la programació i ús del seqüenciador i la PCR a temps real.

A la Núria Gaju, del departament de Genètica i de Microbiologia de la UAB, per facilitar l'ús del microscopi confocal.

A en Lluís Banyeras, del departament de Biologia de la UdG, pels consells i ajuda en la utilització del Citòmetre de flux.

A les meves companyes “moleculars”, que m’han resolt infinitat de dubtes, gràcies Maria i Anna.

A l’Eduard i la Nuri, per fer que la feina no només fos feina, a més de grans científics sou unes magnífiques persones.

A tots els companys de Tecnologia d’Aliments, de l’INTEA i d’EQATA. A la Nuri, la Gemma, la Sussi, la Dolors, la Mònica, l’Elena, la Sònia, l’Anna Maria, en David, l’Anna, la Paula, la M. Àngels, en Pere, la Sussana, en Xavi, en Jose Alberto, l’Elena, la Nuri i la Bego. Gràcies a tots.

Finalment a la meva família i en Tescu, per als quals no tinc prou paraules per agrair tot el recolzament que m’han donat sempre. A vosaltres us dedico aquest treball.