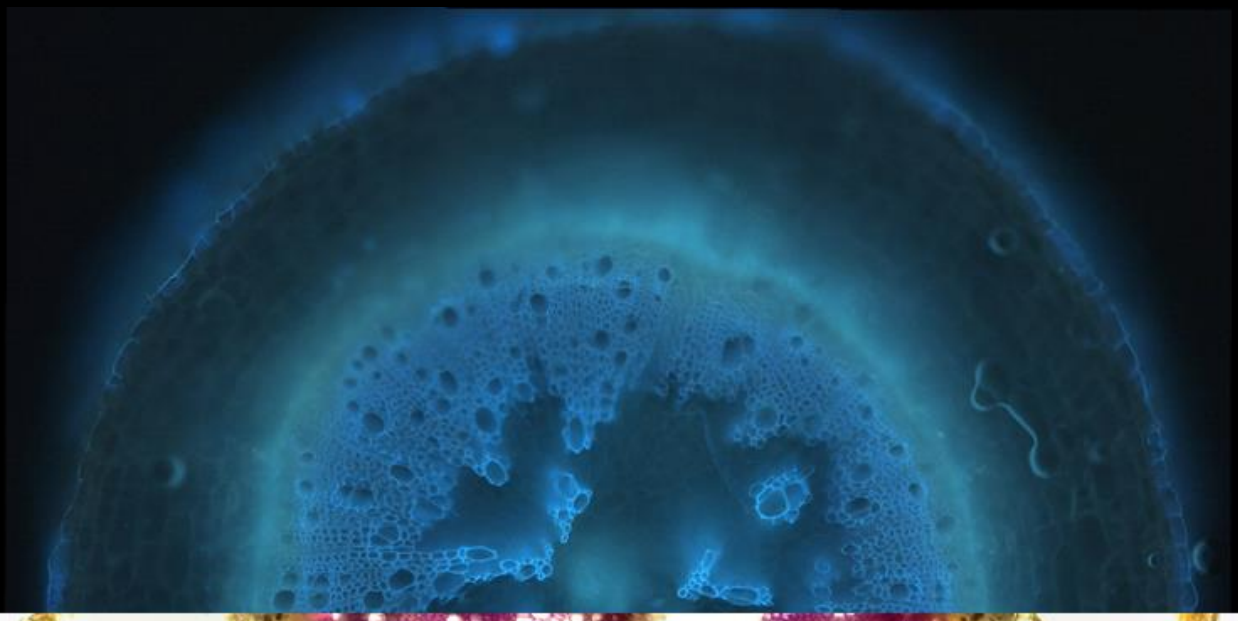
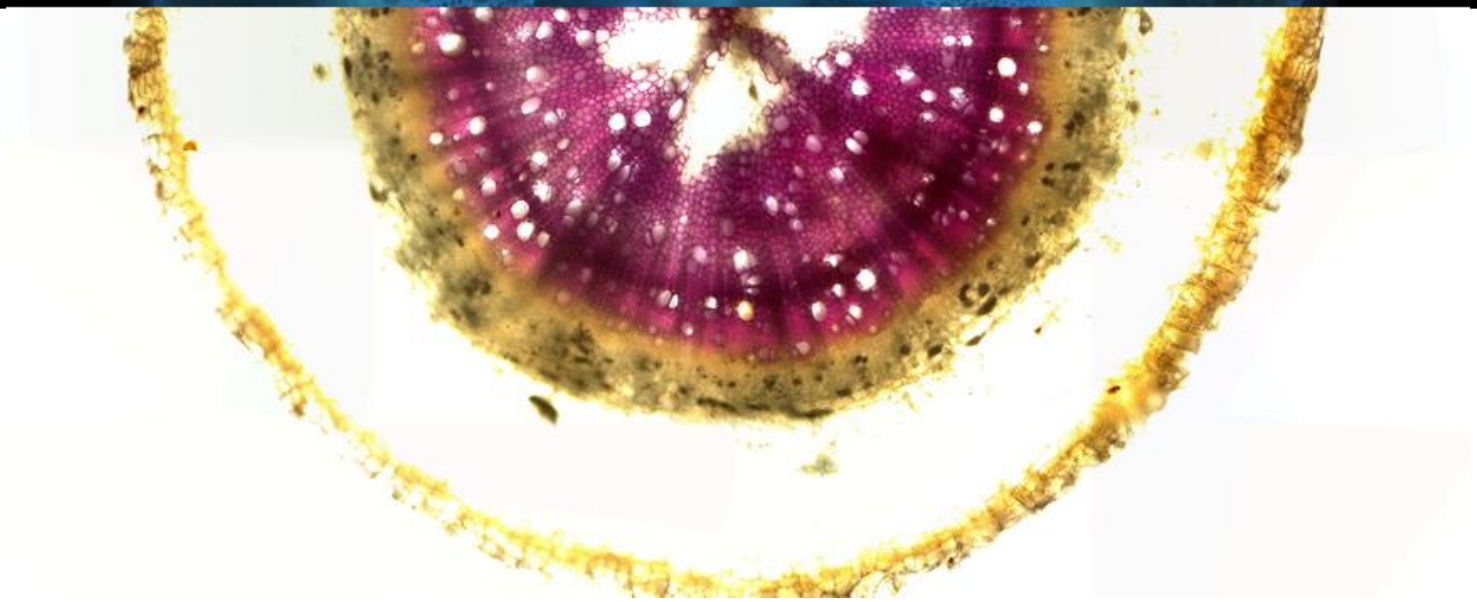
Universitat Autònoma de Barcelona

Insights on the interaction of Ralstonia solanacearum with Solanum dulcamara and Solanum tuberosum

Pau Sebastià Miravet

Insights on the interaction of *Ralstonia solanacearum* with *Solanum dulcamara* and *Solanum tuberosum*





Pau Sebastià Miravet

INSIGHTS ON THE INTERACTION OF *RALSTONIA SOLANACEARUM* WITH *SOLANUM DULCAMARA* AND *SOLANUM TUBEROSUM*

Pau Sebastià Miravet

Sota la direcció de: **Dr. Marc Valls i Matheu**

Tutoritzada per: **Dra. Roser Tolrà Pérez**

PER ACCEDIR AL GRAU DE DOCTOR DINS EL PROGRAMA DE DOCTORAT EN BIOLOGIA I BIOTECNOLOGIA VEGETAL DEL DEPARTAMENT DE BIOLOGIA ANIMAL, BIOLOGIA VEGETAL I ECOLOGIA

Bellaterra, Setembre 2020

The Project that gave rise to these results received the support of a fellowship from “la Caixa” Foundation (ID 100010434). The fellowship code is LCF/BQ/DI17/11620004.

This project has received funding from the European Union´s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 713673

Marc Valls i Matheu, director de la present tesi i professor titular del Departament de Genètica, Microbiologia i Estadística a la Facultat de Biologia de la Universitat de Barcelona, i **Roser Tolrà Pérez**, tutora de la present tesi i professora titular del Departament de Biologia Animal, Biologia Vegetal i Ecologia a la Facultat de Biociències de la Universitat Autònoma de Barcelona, certifiquen:

Que la memòria titulada "**Insights on the interaction of *Ralstonia solanacearum* with *Solanum dulcamara* and *Solanum tuberosum***", presentada per Pau Sebastià Miravet amb la finalitat d'optar al grau de Doctor en Biologia i Biotecnologia Vegetal, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritzen la seva presentació perquè sigui jutjada per la comissió corresponent.

I perquè consti als efectes oportuns, signa la present a Bellaterra, a 25 de setembre de 2020.

Dr. Marc Valls i Matheu	Dra. Roser Tolrà Pérez	Pau Sebastià Miravet
(Director de la Tesi)	(Tutora de la Tesi)	(Doctorand)

Sapere aude

Als meus estimats pares

Agraïments/Acknowledgements

Encara recorde aquell setembre de 2014 quan vaig entrar al teu despatx demanant-te consell per veure la millor opció d'on fer un màster a l'estranger dins de l'àmbit de la biologia vegetal. Cap a Wageningen que vam anar, gràcies en part a tu **Marc**. També recordaré sempre que un novembre de 2016 vaig rebre un correu teu per tal d'informar-me sobre una possible beca (que en aquell moment pensava que no aconseguiria). Gràcies per totes les petites empentes que m'has anat donant durant tots aquests anys i per haver-me ajudat a créixer com a persona i com a científic. **Núria**, tot el meu agraïment per tu també, tot i no ser la meva supervisora directa, m'has ajudat moltíssim amb la teva forma de ser, les teves grans idees i la manera d'encarar els problemes.

To every and single one in Biobact lab, you are the best team ever, far beyond the impact factor in our publications! Començaré amb **Marina**, la primera persona que em va haver d'aguantar i que em va transmetre tot allò que sabies amb una gran humanitat! Gràcies per tot. Jo també recorde els primers mesos al lab i cóm per poc em carregue tots els aparells...**Marc**, sóc feliç d'haver-te conegut i d'haver compartit aprenentatges amb tu, a seguir disfrutant. **Roger**, qui ens anava a dir quan ens vam conèixer a Wageningen que després hauríem de treballar tant junts. Ha sigut un plaer aprendre bioinformàtica amb tu, eres un gran treballador i un molt bon company! No me n'oblide del sopar que farem quan ens publiquen el transcriptoma... **Eugenia**...what to say? I am speechless with you my dear, people like you are the ones that make it worth it to live. I feel we've been like brothers since day 1...and this is gonna be forever don't expect me to leave that easily. **Joselito**, qué sería de nosotros sin las risas? No hace falta que te diga nada más, tu me entiendes. Te espero al salir del doctorado para montarnos algo! **Anurag**, you are simply another level of person! I feel so lucky to have had the opportunity to share so many good (and not as good) moments together. All the best for your last months of PhD thesis, you'll nail it, I am completely sure! **Alex**, empecemos por el final. Sé con toda mi alma que serás un gran padre, tu hijo no sabe el tesoro de padre que se lleva. Gracias por abrirte conmigo desde el minuto 1, por esas conversaciones sin tapujos y por la ayuda cuando peor estaba. **Saúl**, siempre recordaré nuestras esquiadas, pero sobre todo recordaré tu vista fija en el móvil mientras todos hablábamos y tu siempre leyendo papers! Eres un crack, mucha suerte por Suecia y espero que puedas volver pronto a Ecuador. **Nerea**, desgraciadament entre el coronavirus i que ja estava acabant la tesi no hem compartit massa temps al laboratori. Tot el que conec de tu m'agrada, espere que poguem seguir la nostra amistat en el futur. **Weiqi**, all my best for your thesis, don't step back when things look ugly, you'll do it all right! To all former membres of the Biobact lab, it was a big big pleasure to share

our lives together: **Ujjal, Liang Yang, Jonathan, Agnese....**Moltes gràcies també a tu **Montse Capellades**, que sempre tens un moment per ajudar a tothom i sempre amb bon cor.

A ti **Nacho**, por dejarme ser tu amigo, explicarme tus sentimientos aún cuando tu no querías, por atreverte a empezar un pequeño huerto conmigo y enseñarme todo lo que sabes. Espero haberte enseñado alguna cosa pequeño tirabeque. **Unai, Pol i Carlos**, moltes gràcies per aguantar-me sempre que anava al vostre laboratori a molestar i xarrar un poc. Molts ànims, us trobaré a faltar! **Carlos y Tamara**, muchas gracias por todas las conversaciones, discusiones científicas y por los consejos malagueños. Espero que el bebé esté genial porque tiene unos padrazos increibles!

No vull oblidar-me tampoc del gran grup de treballadors que tenim al CRAG i que ens ajuden en el nostre dia a dia i que són tan importants com qualsevol altre científic. Gràcies primer a totes les dones de la neteja, per tenir sempre bon humor i ajudar-nos a tenir un poc més net i ordenat el centre. Gràcies al servei d'hivernacles, en especial a **Sergio, Glòria i Alejandro**, amb qui he tingut més relació i que ofereixen el millor d'ells mateixos pel correcte funcionament del CRAG. No me olvido de ti, **Elena**, por todos esos ratos juntos en *in vitro* contándonos la vida.

Però la vida durant el doctorat ha sigut molt més que les hores passades i viscudes amb intensitat al CRAG. Només començar el doctorat i gràcies al grup de doctorands de la Caixa, he pogut conèixer gent brillant que fa recerca en àmbits d'allò més diversos, i amb una qualitat humana molt major si cab que la seva qualitat científica. Els importants ja sabeu qui sou, d'alguns me n'ha tocat aprendre, pel bo o pel dolent, i amb altres me n'aniria a la fi del món amb ells. No vull desaprofitar aquest espai per agrair a tota la gent que ha treballat en la Caixa per nosaltres: **Gisela, Emilia...**una abraçada molt forta!

Durant tot aquest temps, els amics de sempre, han continuat sent els amics de sempre. Companys de pis immillorables (**Ignacio, Júlia, Adrian, Oleguer, Laia, Maha, Andrea**), els amics de Castelló que continuen allí des d'abans de tindre memòria (**Luis, Carlos, Carlos, Lucas, Pau, Álvaro, Óscar, César, Eduard....**). A vosaltres dos, **Marta i Pau,** que heu estat a tope des de fa 5 anys ja...però també a la resta de gent de Wageningen, allà on va començar aquesta aventura. Nova gent que s'ha anat incorporant i que pareix que estaran una bona temporada amb nosaltres (**Paula, Irene, Anna, Raquel, Andrea, Maria, Laura, Tágara, Charete...**). Els amics de la Llull que mai fallen i que fan que Barcelona siga un poc més casa (**Joan, Juan Fran, Pere, Lluc, Esteve, Nil, Enric, Andreu, Adrià....**).

La meva família de Castelló que tot i que no la puc veure tot el que jo voldria, representa una part molt i molt important del que jo sóc, del que la gent diu: Allò és el que ens agrada de Pau Sebastià Miravet!

Una especial menció per tu **iaia**, que has aguantat la família des que tens 18 anys, separant-te de la teua i donant amor, moltes vegades a canvi de res. Sé que **l'abuelo Javier, l'abuela Matilde i l'abuelo Enrique** estarien feliços de saber que un dels seus nets ha obtingut un doctorat, però segur que estarien encara més feliços de saber que un doctorat no és allò que m'ha fet millor persona. **Papà, Mamà**, m'agradaria ser encara millor escrivint per poder regalar-vos les paraules que vos mereixeu, perdoneu-me per no tindre paraules suficients per vosaltres. Sou un exemple com a pares, però també us he tingut com a amics quan ho he necessitat i també m'heu deixat equivocar-me per prendre les meues pròpies decisions. Per últim no voldria deixar-me a una gran persona que he conegut fa 5 mesos, i que ha fet que l'esforç final de la tesi juntament amb l'estiu més estrany de les nostres vides, hagen sigut molt molt millors. Gràcies **Maria José**! Vos estime moltíssim a tots. Sóc el que sóc, també gràcies a tot el que heu anat fent per mi.

SUMMARY IN ENGLISH

Ralstonia solanacearum is one of the most destructive bacterial plant pathogens worldwide. It causes bacterial wilt on more than 200 plant species, among them economically important crops. A big effort has been done during the last decades in order to identify the most relevant virulence factors of this pathogen, but a comprehensive and dynamic understanding of the gene expression profile of these virulence determinants along the infection process was lacking. Also, different groups have identified wild reservoir hosts of *R. solanacearum*, which has helped in broadening the knowledge of how this bacterium completes its complex life cycle. However, the information regarding the interaction between *R. solanacearum* and its reservoir hosts was very scarce.

In this thesis, we have tackled the first challenge by establishing three different infection stages of *R. solanacearum* UY031 strain in potato: apoplast, early xylem and late xylem. The bacterial transcriptome along the infection process and its analysis revealed how *R. solanacearum* activates or represses specific virulence genes to hijack plant defences as well as the use of bacterial metabolic pathways to facilitate the colonization of different plant tissues during the infection. We underscore the fact that *R. solanacearum* induces the expression of most of its type III effectors during the xylem phase. In addition to that, different types of motility are regulated and associated to different plant environments and last but not least, that *R. solanacearum* largely depends on the activation of different nitrogen metabolism genes to adapt to the hypoxic xylem vessels.

The second big challenge of this thesis has been to understand how *R. solanacearum* interacts with its reservoir host *Solanum dulcamara*. After analysing the infection process using different inoculation methods, we concluded that *S. dulcamara* it is not only a reservoir host but also a tolerant host of *R. solanacearum*, since similar bacterial loads are found compared to other susceptible hosts, but the bacterial wilt symptoms are much lower. We learned that *S. dulcamara* presents a very stable xylem lignification, which is not impaired upon bacterial infection, partly explaining this tolerant phenotype. Lastly, we found out that *S. dulcamara* tolerance against *R. solanacearum* is even more clear at cooler temperatures, underscoring the importance of the environment when investigating plant pathogen interactions.

In summary, this work provides both a solid ground for *in planta R. solanacearum* functional and characterization studies of different virulence factors, as well as an insight of how the bacterium behaves inside its reservoir tolerant host *S. dulcamara*.

RESUM EN CATALÀ

Ralstonia solanacearum és un dels patògens de plantes bacterians més destructius arreu del món. Provoca el marciment bacterià en més de 200 espècies vegetals, entre elles cultius d'alta importància econòmica. Grans esforços s'han dut a terme durant les últimes dècades per tal d'identificar els factors de virulència més rellevants d'aquest patogen, però faltava una comprensió més detallada i dinàmica sobre els perfils d'expressió gènica dels determinants de virulència al llarg del procés d'infecció. Així també, diferents grups de recerca han identificat hostes salvatges de *R. solanacearum*, la qual cosa ha ajudat a ampliar el coneixement de cóm aquest bacteri completa el seu cicle de vida complex. No obstant això, la informació relacionada amb la interacció entre *R. solanacearum* i els seus hostes reservori era molt escassa.

En aquesta tesi, hem abordat el primer repte mitjançant l'establiment de tres estadis diferents d'infecció de la soca UY031 de *R. solanacearum* en patatera: l'apoplast, xilema inicial i xilema tardà. El transcriptoma bacterià al llarg de la infecció i els seu posterior anàlisi van revelar com *R. solanacearum* activa o reprimeix gens de virulència específics per tal de manipular les defenses de la planta així com l'ús de vies metabòliques bacterianes per facilitar la colonització dels diferents teixits vegetals durant la infecció. Destaquem el fet que *R. solanacearum* indueix l'expressió de la majoria dels efectors de tipus III durant la fase del xilema. A més a més, diferents tipus de motilitat bacteriana estan regulades i associades a diferents ambients d'infecció de la planta i per últim, que *R. solanacearum* depèn de l'activació de diferents gens del metabolisme del nitrogen per adaptar-se a ambients d'hipòxia al xilema.

El segon gran repte d'aquesta tesi ha sigut comprendre cóm *R. solanacearum* interacciona amb el seu hoste reservori *Solanum dulcamara*. Després d'analitzar el procés d'infecció utilitzant diferents mètodes d'inoculació, vam concloure que *S. dulcamara* no és només un hoste reservori però també un hoste tolerant de *R. solanacearum*, perquè quantitats similars de bacteri es troben comparat amb altres hostes susceptibles, però els símptomes de marciment bacterià són més baixos. Hem aprés que *S. dulcamara* presenta una estructura lignificada al xilema molt estable que no disminueix després de la infecció, la qual cosa explica parcialment el fenotip tolerant observat. Per últim, hem esbrinat que la tolerància de *S. dulcamara* contra *R. solanacearum* és més marcada a temperatures més fresques, destacant la importància de l'ambient quan s'investiguen interaccions entre plantes i patògens.

En resum, aquest treball proporciona tant un terreny sòlid per estudis de caracterització de diferents factors de virulència de *R. solanacearum in planta*, com també una aproximació del comportament del bacteri dins del seu hoste reservori tolerant *S. dulcamara*.

INDEX

INTRODUCTION

1.1 PLANT DISEASES AS A THREAT TO AGRICULTURE: THE CASE OF BACTERIAL WILT

1.1.1 Agricultural impact caused by plant pathogens

Plants are sessile organisms that live in fluctuating environments confronting different stresses, which prevent them from attaining their full growth and yield potential (Bray et al., 2000). Stresses are classified into abiotic (e.g. heat, cold, drought, salinity, nutrient stress) (W. X. Wang et al., 2003), and biotic (e.g. infection by fungi, bacteria, viruses, nematodes, insects), and both combined have a huge impact on agricultural yields worldwide. Since biotic stresses are caused by living organisms, this adds a new layer of complexity to the plant biotic stresses compared to abiotic ones. From the plethora of different organisms that interact with plants, some of them are beneficial (e.g. insect pollinators, nitrogen-fixing bacteria and fungal mycorrhizae), while other organisms are harmful and threaten the plant integrity (Van Dam, 2009). Investigate how crop plants thrive and survive the diversity of pathogens is crucial to understand the complexity of pathogen life cycles in nature and improve the agricultural system, boosting crop productivity.

Annually, all plant pathogens combined (i.e. parasitic weeds, animal pests, fungi, oomycetes, bacteria and viruses) cause approximately 30-40% of production losses worldwide for the major staple crops (i.e. wheat, rice, maize, potatoes, soybean and cotton) (Oerke, 2006). Bacterial plant pathogens cause around 15% of the total crop losses, although there is a huge variety in their host ranges and the extent of severity of the diseases they cause (Oerke & Dehne, 2004). Different bacterial plant pathogens infect the most important staple crops such as *Erwinia stewartia* (maize), *Xanthomonas oryzae* (rice), *Xanthomonas translucens* pv. *undulosa* (wheat), *Ralstonia solanacearum* (potato) and *Xanthomonas axonopodis* pv. *manihotis* (cassava) (Strange & Scott, 2005). Based on the risk on crop production and their scientific importance, a list of the 10 most relevant bacterial plant pathogens was elaborated, such as *Pseudomonas syringae* (bacterial speck), *Ralstonia solanacearum* (bacterial wilt) and *Erwinia amilovora* (fire blight) among others (Mansfield et al., 2012) (Table 1, Figure 1).

Table 1. Most relevant bacterial phytopathogens. Adapted from (Mansfield *et al.*, 2012).

Pathogen	Plant host	Disease
Pseudomonas syringae pathovars	Tomato, bean, olive, oats	Bacterial speck
Ralstonia solanacearum	Potato, tomato, eggplant	Bacterial wilt
Agrobacterium tumefaciens	Grapevines, nut trees, stone fruits	Crown gall
Xanthomonas oryzae pv. *oryzae*	Rice	Bacterial blight
Xanthomonas campestris pathovars	Pepper, tomato, brassicas	Black rot, Bacterial spot
Xanthomonas axonopodis pv. *manihotis*	Cassava	Cassava bacterial blight
Erwinia amylovora	Apple, pear, raspberry	Fire blight
Xylella fastidiosa	Gravines, coffee, *Prunus* spp., *Citrus* spp.	Pierce's Disease, Almond leaf scorch, Citrus variegated chlorosis
Dickeya (*dadantii* and *solani*)	Potato	Black leg, soft rot
Pectobacterium carotovorum (and *P. atrosepticum)*	Potato	Soft rot, black leg

Figure 1. Disease symptoms caused by the most relevant bacterial phytopathogens.
1) *Pseudomonas syringae* - bacterial speck of tomato (picture from K. Loeffler and A. Collmer). 2) *Ralstonia solanacearum* – bacterial wilt of tomato (picture original from this work). 3) *Agrobacterium tumefaciens* – crown gall of blueberry (picture from Kado 2002). 4) *Xanthomonas oryzae* pv. *oryzae* – bacterial blight of rice (picture from Sun *et al*. 2016). 5) *X. campestris* – bacterial spot of cabbage (picture from Mansfield *et al.*, 2012). 6) *X. axonopodis* pv. *manihotis* – bacteria blight of cassava (picture from Mansfield *et al*., 2012). 7) *Erwinia amylovora* – fire blight of apple (picture from Mansfield *et al*. 2012). 8) *Xylella fastidiosa* – Pierce's disease of plum (picture from Thorne *et al.* 2006). 9) *Dickeya dadantii* – soft rot of potato (picture from Mansfield *et al.* 2012). 10) *Pectobacterium carotovorum* – black leg of potato (picture from Manfield *et al*. 2012).

1.1.2 Disease triangle

In plant-pathogen interactions, there are three main players that participate: the plant, the pathogen and the environment. Plant diseases successfully occur in nature only when there is a coordination of these three factors: a virulent pathogen, a susceptible plant host and a permissive environment. They can interact in the commonly known disease triangle, a concept formally integrated into plant pathology by George McNew in 1960, with the intention of creating a tool to predict, limit and eventually control epidemics (McNew, 1960). Interestingly,

the three main actors involved in the disease triangle are further divided into: severity of the physical environment, duration of the infection period, prevalence of the pathogen, virulence of the pathogen, the age/maturity of the host plant and its inherent susceptibility to disease (Scholthof, 2007) (Figure 2). Newer versions of the disease triangle include "time" or "man" as a fourth factor, transforming the triangle into a pyramid or tetrahedron, and providing a more accurate description of the complexity of occurrence and are maintained through time (Francl, 2001).

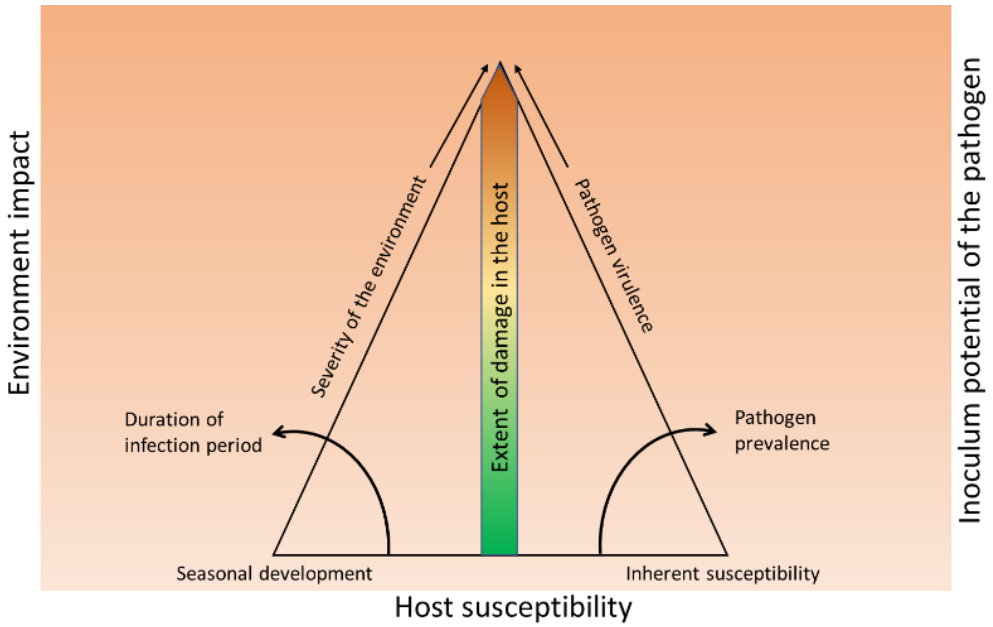


Figure 2. Disease triangle. The outcome of a plant – pathogen interaction is determined by three main factors: the pathogen, the plant host and the environment. The more permissive the environment, the longer the infection period, the higher the pathogen virulence and the pathogen prevalence and the higher inherent host susceptibility and seasonal development, the more successful the disease will develop in the plant host and the higher the extent of the damage (gradient vertical arrow). Figure adapted from Scholthof 2007.

1.1.3 Climate change and emerging bacterial phytopathogens

Since the environment is one of the main factors in order to have a successful plant disease, the current climate change scenario opens up new uncertainties and risks in plant epidemiology (Garrett et al., 2006). Although the effects of climate change will tend to be different for each pathosystem and location, temperature increases are predicted to promote the geographical spread of pathogens to new areas, bringing pathogens into contact with new potential plant hosts (Baker et al., 2000), as well as providing the ground for pathogen hybridization (Brasier, 2001). Moreover, climate change will result in longer seasons, giving more time for pathogens to reproduce and evolve (Tapsoba & Wilson, 1997; Waugh et al., 2003). However, the total effect that climate change will have on the disease outcome will

depend on how the temperature raise affects the encounters between plants and pathogens, the virulence and aggressiveness of pathogens, and the changes in plant host resistance (Garrett et al., 2006). Some bacterial plant pathogenic species such as *Ralstonia solanacearum*, *Acidovorax avenae*, and *Burkholderia glumea*, have optimal growing temperatures above 30ºC and some can still optimally grow up to 41ºC, which suggests that the diseases caused by these pathogens will be favoured by global warming (Schaad, 2008). Temperate regions like Central Europe or USA that were pathogen-free before this climate change scenario, are now at big risk of becoming endemic areas of these new emerging plant pathogenic bacteria (Anderson et al., 2004; Kudela, 2009). Therefore, it is of crucial interest to study the life cycles of these bacterial pathogens to gain knowledge for improving the agricultural system.

1.1.4 The case of bacterial wilt

Ralstonia solanacearum is one of the most devastating bacterial plant pathogens (Mansfield et al., 2012), causing bacterial wilt disease, which was originally described in 1896 by E. F. Smith (Smith, 1896). *R. solanacearum* is a soil-borne gram-negative β-proteobacterium with an unusually wide host range, infecting more than 200 plant species, among them economically important crops (e.g. tomato, potato, eggplant, banana, peanut, olive) but also model plant species in research (i.e. *Arabidopsis thaliana* or tobacco) (Hayward, 1991). Due to its large phenotypic and genotypic diversity, *R. solanacearum* is now classified as a species complex (RSSC), including two related species: *R. syzygii* and the Blood Disease Bacterium (Prior & Fegan, 2005). The RSSC has been further subdivided into five races according to the host range, into five biovars based on which sugars and alcohol carbohydrates can metabolize, and into four phylotypes (I-IV) and 23 sequevars based on their endoglucanase sequence (Prior & Fegan, 2005) (Figure 3). Although *R. solanacearum* natural habitat is the tropical and subtropical regions, phylotype II-B1 strains have adapted to colder temperatures and caused important bacterial wilt outbreaks in Europe and the USA (Elphinstone, 1996; Janse et al., 2004) (Figure 3).

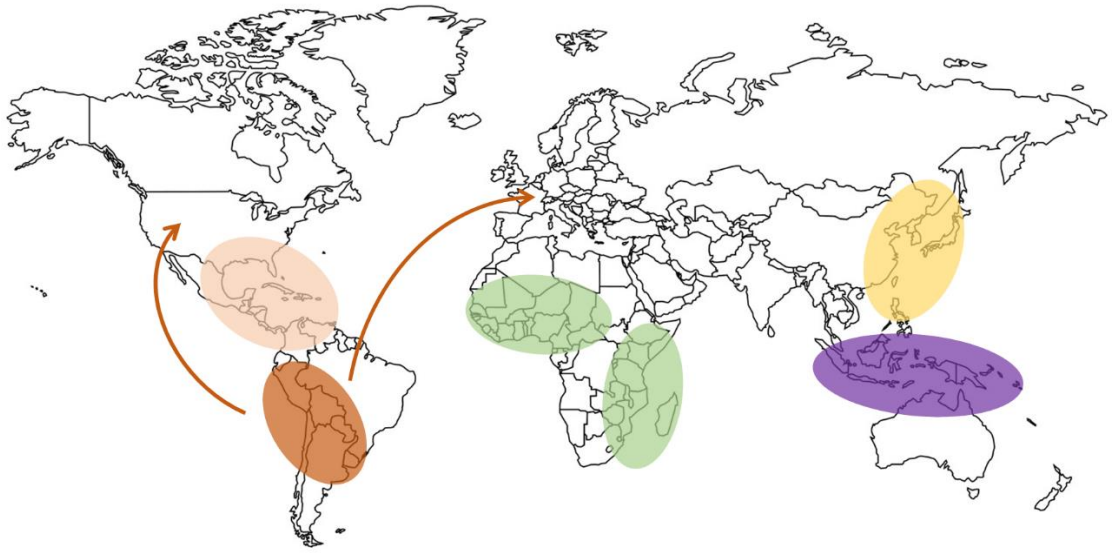


Figure 3. Geographical distribution of the *R. solanacearum* species complex. Coloured circles represent regions were *R. solanacearum* is endemic. Brown arrows represent areas where phylotype II-B1 strains have emerged. Colours indicate the prevalent phylotype in each endemic region: yellow (phylotype I), light brown (phylotype IIA), brown (phylotype IIB), green (phylotype III) and purple (phylotype IV).

Due to its broad host range, its high aggressiveness and the wide geographical distribution, *R. solanacearum* is considered among the most threatening bacterial pathogens (Mansfield et al., 2012). Resistance genes have been considered as the most durable control measure against bacterial wilt, using different wild potato relatives as sources of resistance such as *Solanum phureja* (Sequeira & Rowe, 1969)*, Solanum commersonii* (Carputo et al., 2009) and *Solanum aethiopicum* (Collonnier et al., 2001). In addition to that, tomato cultivars such as CRA 66 and Hawaii 7996, both resistant to bacterial wilt, have been used as resistant rootstock grafted with the scion of the organic tomato cultivar German Johnson, resulting in no bacterial wilt symptoms (Rivard & Louws, 2008). Monogenic resistance to *R. solanacearum* has been also reported in the model plant *Arabidopsis thaliana* with the interaction between the plant R gene *RRS1-R* and the bacterial type III effector PopP2 (Deslandes et al., 2003). However, most reports describing resistance to *R. solanacearum* involve polygenic traits such as the case for the wilt potato relative *S. commersonii* (M. González et al., 2013), a quantitative trait loci containing several R genes in the model legume *Medicago truncatula* (Ben et al., 2013), several quantitative trait loci in two tobacco breeding lines (Qian et al., 2013), as well as one major dominant resistance gene named ERs1 and one quantitative trait loci involved in delay of disease progress in eggplant (Lebeau et al., 2013). Unfortunately, the fact that most resistance cases have been associated to quantitative trait loci, makes it difficult to introduce it to commercial crop varieties without the introduction of undesired linked genes (T. Denny, 2006).

Traditional strategies to cope with different bacterial plant disease included antibiotics and copper-based compounds (Zaumeyer, 1958). Nowadays, alternative control measures to deal with bacterial wilt in the field include targeted chemical compounds (Puigvert et al., 2019; Sundin et al., 2016), plant systemic defence inducers (Pradhanang et al., 2005), and specific *R. solanacearum* antimicrobials (J. K. Hong et al., 2016). In countries were bacterial wilt is not endemic yet, the most common control measure is avoiding introduction of contaminated seed tubers, while in endemic areas the common practices are crop rotation, and control of weed reservoirs and irrigation water (T. Denny, 2006).

1.2 DIVERSITY OF LIFE CYCLES IN BACTERIAL PHYTOPATHOGENS

1.2.1 Life cycles of bacterial phytopathogens

Bacterial plant pathogens have different mechanisms to enter, colonize and harm the plant host, as well as different strategies to survive outside the susceptible plant. Understanding the diversity of life cycles of bacterial phytopathogens is of utmost importance for designing better short and long-term control strategies (Narayanasamy, 2017). For this purpose, the life cycles of some of the most relevant or model bacterial plant pathogens will be described below: *Xanthomonas oryzae* pv. *oryzae, Pseudomonas syringae* pv. *tomato*, *Xylella fastidiosa* and *Ralstonia solanacearum*.

1.2.1.1 *Xanthomonas oryzae* pv. *oryzae*

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the causal agent of the bacterial blight, one of the most destructive diseases of rice (*Oryza sativa*) (Niño-Liu et al., 2006). *Xoo* is a rod-shaped, gram-negative species that produces large amounts of extracellular polysaccharide (EPS), which forms droplets of bacterial exudates in the plant leaf, providing protection from desiccation and aiding in wind- and rain-borne dispersal of the bacteria (Swings et al., 1990). *Xoo* is an obligate aerobic organism, not spore-forming, with an optimal growth temperature of 25-30ºC (Niño-Liu et al., 2006). The pathogen is endemic in both tropical and temperate areas (Mew, 1993). *Xoo* enters the rice leaf through hydathodes at the leaf tip and leaf margin (Ou, 1985), bacteria multiply in the intercellular spaces of the underlying epithem, then enter and spread into the plant through the xylem (Noda & Kaku, 1999). Once inside the vasculature, it moves vertically along the leaf primary veins but it can also travel to lateral veins, colonizing

the whole leaf within a few days, finally forming exudates on the leaf surface, a trait of a late disease stage and a source of secondary inoculum (Mew, 1993) (Figure 4).

Bacterial blight outbreaks mainly occur during the monsoon season (June to September) in south-east Asia and India (Mew, 1993), where wind and rain facilitate bacterial dispersion from infected rice leaves to other hosts such as wild rice species (*O. rufipogon, O. australiensis*) and a number of gramineous weeds (*Leersia* spp. and *Zizania latifolia* in temperate regions and *Leptochloa* spp. and *Cyperus* spp. in the tropics) (Niño-Liu et al., 2006). In temperate regions, *Xoo* can survive in the rhizosphere of weeds of the genera *Leersia* and *Zizania* (Mizukami & Wakimoto, 1969), as well as in the soil for 1-3 months depending on the moisture and acidity (Ou, 1985). During the last two decades, different reports have shown that different *Leersia* spp. such as *Leersia hexandra, Leersia sayanuka, Leersia oryzoides,* and *Leersia japonica*) grow along rice fields and can serve as asymptomatic reservoir hosts of *Xoo* (C. F. González, 1991; Lang et al., 2019; Noda & Yamamoto, 2008).

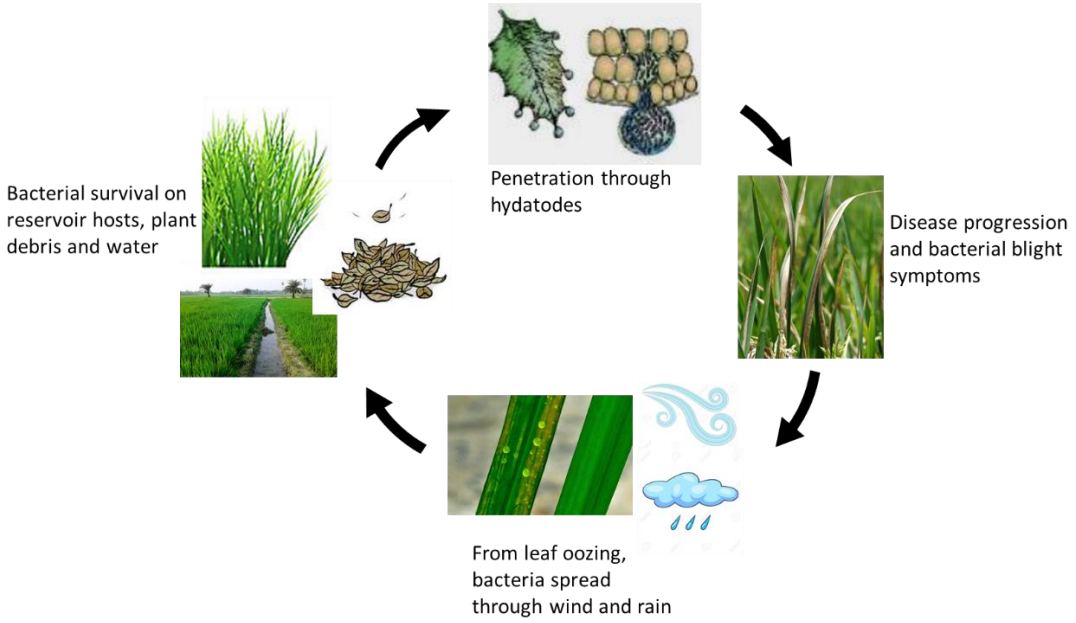


Figure 4. Disease cycle of *Xanthomonas oryzae* pv. *oryzae*. *X. oryzae* pv. *oryzae* enters the leaf through hydathodes, multiplies in the intercellular spaces of the epithem and progressing towards the vasculature. At late disease stages, it forms leaf exudates, which are forms of secondary inoculum spread by rain and wind. The pathogen can also survive on reservoir hosts, plant debris and water.

1.2.1.2 *Pseudomonas syringae* pv. *tomato*

Pseudomonas syringae has been ranked as the most important bacterial phytopathogen due to its high scientific impact as a bacterial phytopathogen model (Mansfield et al., 2012). *P. syringae* spp. can infect a wide range of plant species, although each *P. syringae* species is considered to have a discrete and narrow host range (Xin & He, 2013). *P. syringae* is a gram-

negative hemibiotrophic pathogen, infecting mainly aerial parts of plants such as leaves and fruits (Xin & He, 2013).

Initially, the pathogen has an epiphytic phase in the leaf surface, where it encounters harsh conditions such as rapid changes in temperature, sunlight and moisture (Lindow & Brandl, 2003). Many *P. syringae* strains (e.g. B728a) are good epiphytes and can provide inoculum for subsequent infections, while others (e.g. DC3000) are weak epiphytes but extremely effective at multiplying in the leaf apoplast (Boureau et al., 2002) (Figure 5). In the early 90s, *P. syringae* pathovar *tomato* DC3000 (*Pto* DC3000) was shown to not just infect tomato but also *Arabidopsis thaliana*, which boosted the use of this pathovar as a model for bacterial phytopathogen studies (Whalen et al., 1991). *Pto* is the causal agent of bacterial speck of tomato, which provoke necrotic lesions on leaves, stems, and fruit of tomato plants (Goode & Sasser, 1980). *Pto* can survive in infected tomato seeds (Devash et al., 1980), as well as in asymptomatic weed hosts, serving as reservoirs for future infections (Schneider, 1977). From *Pto* epiphytic populations on leaf surfaces, the pathogen fluxes upward through the air to the atmosphere (Constantinidou et al., 1982; Sands et al., 1982), where it can fall out to different new environments (Figure 5).

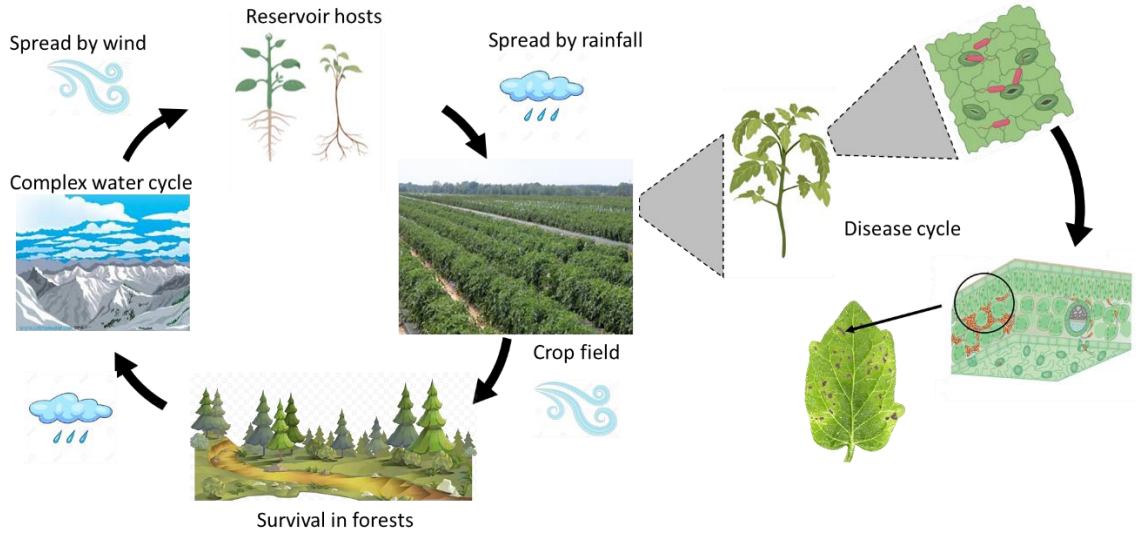


Figure 5. Disease cycle of *Pseudomonas syringae* pv. *tomato*. On the left, complete life cycle of *P. syringae* pv. *tomato*. The pathogen moves from the crop to other ecological niches such as forests, reservoir host, boosted by a complex water cycle and spread by wind and rain. On the right, schematic representation of the pathogen disease cycle in tomato. The bacterium enters the tomato leaf through the stomata, heavily multiplies in the leaf apoplast causing visible necrotic spots on the leaf surface. Disease cycle in the right adapted from Xin *et al*. 2013.

1.2.1.3 *Xylella fastidiosa*

Xylella fastidiosa is a gram-negative xylem-limited gamma-proteobacterium with a wide host range (Wells et al., 1987). To date, *X. fastidiosa* is known to infect around 350 plant species,

which is increasing due to research on new epidemics in the Mediterranean (Report, 2016). This vascular phytopathogen was first described in 1892 by Newton Pierce to cause devastating epidemics on grapevine in California, and the disease was called Pierce's disease after him (Pierce, 1892). Due to the huge impact that *X. fastidiosa* had on citrus in Brazil, it was the first plant pathogen whose genome was sequenced (Simpson et al., 2000). Quite unexpectedly, several outbreaks of *X. fastidiosa* were detected in olive trees from 2013 onwards in different locations of Italy (Saponari et al., 2013), southern France (Denance et al., 2017) and Spain (Olmo et al., 2010), which increased the research interest on the bacterial pathogen.

Interestingly, *X. fastidiosa* is an obligatory colonizer of plants and insect vectors (Almeida & Nunney, 2015; Wells et al., 1987). Plant colonization is limited to xylem vessels, which leads to reduced sap flow (Baccari & Lindow, 2011). Surprisingly, *X. fastidiosa* low cell density upregulates plant colonization and movement, whereas at high cell densities, the bacterium switches to an insect acquisition and colonization phenotype (Chatterjee, Wistrom, et al., 2008) (Figure 6). Insect vectors visually discriminate plants with Pierce's disease symptoms, which occur at high *X. fastidiosa* cell densities (Daugherty et al., 2011). Therefore, the cell-density-dependent phenotype switch in the pathogen is an adaptation that allows the bacterium to colonize the plants up to a certain threshold before causing plant symptoms that would detract insect vectors from sucking the xylem sap and spread the bacterium further (Sicard et al., 2018).

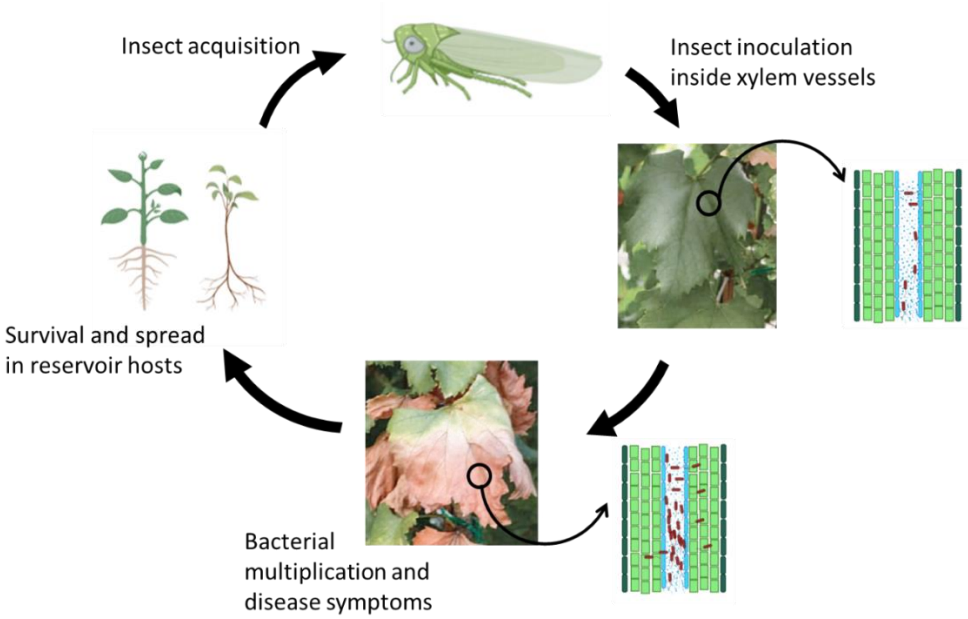


Figure 6. Disease cycle of *Xylella fastidiosa*. *X. fastidiosa* is an obligate xylem pathogen, which heavily multiplies in the xylem vessels of grapevines and is transmitted by insects directly from the xylem sap. *X. fastidiosa* can also survive on reservoir hosts. Disease symptom pictures from Picavoli *et al*. 2017.

1.2.1.4 *Ralstonia solanacearum*

As a soil-borne bacterial phytopathogen, *R. solanacearum* survives in the soil until it specifically recognizes the rhizosphere of host plants (Yao & Allen, 2006). Shortly after root surface attachment, *R. solanacearum* starts root colonization either through the elongation zone, root wounds or secondary root emerging points (J. Vasse et al., 1995). Then, bacteria invades the cortical apoplastic spaces, which is the previous step to the colonization of the vascular cylinder and the xylem vessels (Digonnet et al., 2012). Bacterial cell wall-degrading enzymes have an important role in this infection stage by facilitating the entry of *R. solanacearum* into the xylem vessels (Qi Huang & Allen, 2000; Huanli Liu et al., 2005; Tans-Kersten et al., 1998). Few days after reaching the xylem vessels, *R. solanacearum* rapidly multiplies up to 10^{10} CFU/ml (approximately 10^{9} CFU/g tissue) (Jacobs et al., 2012; J. Vasse et al., 1995), which activates the production of exopolysaccharide (EPS) (J. Huang et al., 1995), finally clogging the plant vasculature and killing the plant by impairing the water flow (T. P. Denny et al., 1990) (Figure 7).

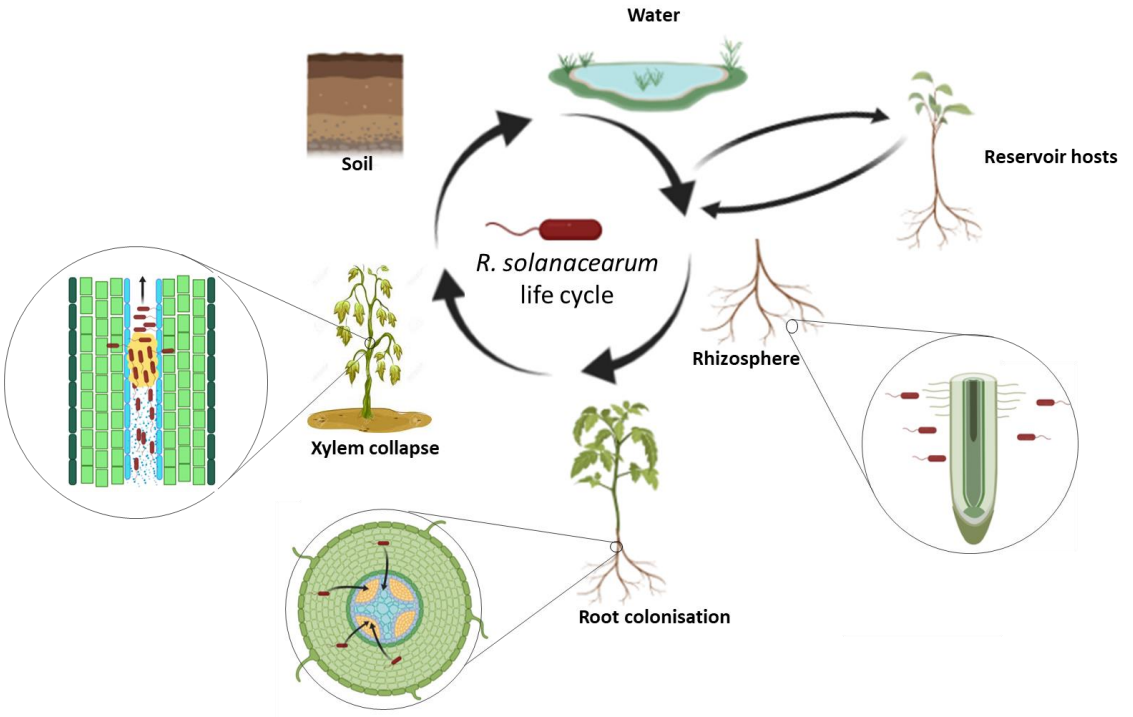


Figure 7. Disease cycle of *Ralstonia solanacearum*. *R. solanacearum* is a soil-borne pathogen that can survive for long periods in soil and waterways. Once it finds the roots of a plant host, it enters through wounds or secondary roots. The root apoplast is the first colonization stage, until it crosses the Casparian strip and enters the vasculature. There, it heavily multiplies, producing the exopolysaccharide, which leads to xylem vessel clogging and blocking water upstream. Once the plant dies, the bacterium returns to the soil, where it can find reservoir hosts that can asymptomatically infect and use them as overwintering shelters.

Once the plant is completely wilted, *R. solanacearum* returns to the soil, where it can shift to a saprophyte lifestyle by feeding from plant debris (Belén Álvarez et al., 2007; Graham et al., 1979). At this stage of its life cycle, *R. solanacearum* inactivates most of its metabolism, acquiring a quiescent state called the reversible viable but non culturable state (VBNC), which allows the bacteria to overcome nutrient starvation for long periods and reactivate their metabolism after encountering a host rhizosphere (Grey & Steck, 2001). Besides surviving in soil for long periods, *R. solanacearum* can also survive in water streams, through which it can spread and infect further locations (J. C. Hong et al., 2008). Especially important is the fact that phylotype II-B1 strains can survive at cold temperatures and spread to previously pathogen-free areas (e.g. Europe) (Caruso, Palomo, Bertolini, López, et al., 2005; Elphinstone, 1996; Graham et al., 1979). Although *R. solanacearum* has the ability to survive for long periods in soil and waterways, the pathogen has been identified infecting asymptomatically different hosts growing near rivers or crop fields, providing a shelter for the winter (Hayward, 1991).

1.2.2 Reservoir hosts

Although most studies concerning bacterial phytopathogens focus on the interaction with susceptible crop plants, it is of utmost importance to have a broader picture of other ecological aspects of their life cycles. Most bacterial phytopathogens do not rely on a single host species to complete their life cycles, but they infect other plant species as means to maintain and/or spread their bacterial populations (M. G. Roberts & Heesterbeek, 2020). The concept of reservoir host is sometimes misleading but it is commonly referred to the ability of a plant species to shelter the pathogen and to act as a sustained source of transmission to a target plant population (e.g. susceptible crop) (Haydon et al., 2002; M. G. Roberts & Heesterbeek, 2020) (Figure 8).

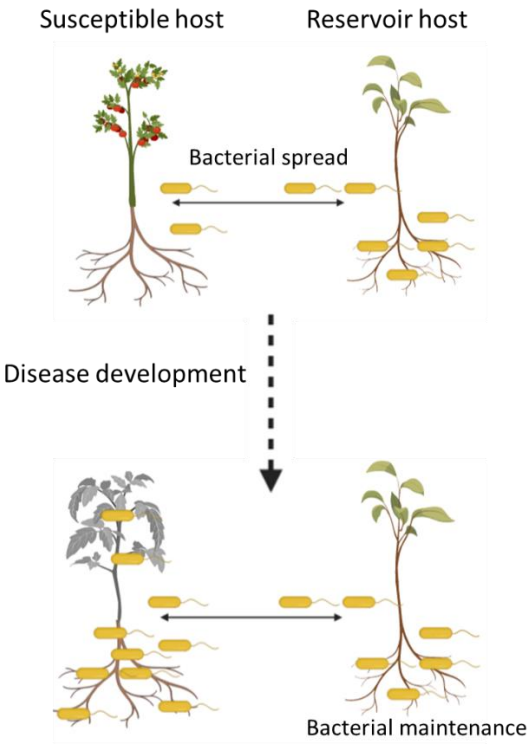


Figure 8. Reservoir host role in plant pathology. Plants that act as reservoir hosts of a certain pathogen must be able to spread the pathogen to a susceptible target host, as well as maintaining a pathogen population asymptomatically.

Wild plant species have been identified as reservoir hosts of the main bacterial phytopathogens previously described. However, due to the ubiquitous presence of many bacterial phytopathogens in different niches, it is important to keep in mind that it is very likely that most if not all pathogens might have reservoir hosts, even though they have not been reported yet. For instance, as mentioned above, it was found that different species from the genus *Leersia,* closely related to rice, could serve as reservoir hosts for *X. oryzae* pv. *oryzae* (C. F. González, 1991; Noda & Yamamoto, 2008). It was reported that different isolates of *X. oryzae* pv. *oryzae* could asymptomatically infect different *Leersia* spp., that reinoculated bacteria to rice and cause bacterial blight disease, and that most *Leersia* spp. individuals were found in water canals next to rice fields, confirming that *Leersia* was a reservoir host of the pathogen and the primary source of inoculum for further infections (C. F. González, 1991; Noda & Yamamoto, 2008).

Similarly, the causal agent of bacterial speck in tomato, *P. syringae* pv. *tomato*, was isolated from leaves and roots of asymptomatic plants (e.g. *Beta vulgaris, Brassica campestris, Brassica nigra, Matricaria matricarioides*) growing on commercial tomato fields, thus serving as sources of inoculum to infect susceptible tomato plants (Schneider, 1977). Interestingly, this study also found out that *P. syringae* pv. *tomato* could establish resident populations inside tomato leaves without causing bacterial speck under warm and dry conditions, but once the humidity

was recovered, symptoms developed in few days (Schneider, 1977). Due to the agronomic and scientific importance of *P. syringae* spp., the ecology of these bacteria has been widely studied, and non-agricultural niches linked to the water cycle (e.g. clouds, fresh snow, rainfall, snowpack) have been underscored as important means of spreading the pathogen to new locations (Morris et al., 2013).

X. fastidiosa, is also able to asymptomatically infect many different plant species, which facilitates its introduction to new environments (Sicard et al., 2018). The presence of asymptomatic reservoir hosts of *X. fastidiosa* appears to be important in the disease epidemiology of Pierce's disease in California. It was shown that many different perennial plant species where *X. fastidiosa* insect vectors overwintered could also harbour populations of the bacterial phytopathogen asymptomatically (Purcell & Saunders, 1999). The list of plant species in northern California that are described as reservoir hosts of *X. fastidiosa* include: big leaf maple (*Acer macrophyllum*), California buckeye (*Aesculus californica*), California blackberry (*Rubus ursinus*), coast live oak (*Quercus agrifolia*), elderberry (*Sambucus mexicana*), French broom (*Genista monspessulanus*), periwinkle (*Vinca major*), valley oak (*Quercus lobate*), and the grape root-stock *Vitis rupestris* (Purcell & Saunders, 1999). Interestingly, most of the tested plant species supported *X. fastidiosa* growth, although with both reduced multiplication and systemic movement throughout the plant compared to grapevine. Periwinkle and French broom, both of them found next to grapevine fields, support high populations and systemic movement of *X. fastidiosa,* which make them good reservoir hosts of the bacterial pathogen (Purcell & Saunders, 1999).

R. solanacearum has one of the widest host ranges amongst the most devastating bacterial phytopathogens as mentioned before. This unique large host range suggests that *R. solanacearum* would be easily found in other wild plants apart from the susceptible crop hosts. In the last 50 years, different reservoir hosts have been reported for *R. solanacearum*. Back in 1970s, Olsson *et al*., linked the appearance of wilted potato plants with the presence of *Solanum dulcamara* in different farms in Sweden (Olsson, 1976). Interestingly, during the coldest months, *R. solanacearum* phylotype II-B1 was not found in irrigation water, but it was identified in the xylem of adventitious roots of *S. dulcamara* (Olsson, 1976). By the same time, another closely related plant species, *Solanum nigrum*, was also found surrounding potato fields in Australia carrying *R. solanacearum* (Hayward, 1975). *Solanum cinereum* (Graham & Lloyd, 1978) and stinging nettle (*Urtica dioica*) (Wenneker et al., 1999) were both shown to be perennial natural hosts of *R. solanacearum* phylotype II-B1 and it was demonstrated both under controlled and natural conditions that the infection was successful without any wilting

symptom (Wenneker et al., 1999). A study carried out to identify new weed hosts of *R. solanacearum* from highland Uganda, found out that several wild plant species (e.g. *Amaranthus* spp., *Bidens pilosa, Galinsoga parviflora*) could be used as sources of inoculum to later on infect susceptible potato plants (Tusiime et al., 1997). Moreover, several other European weed species have been identified as potential *R. solanacearum* phylotype II-B1 hosts under artificial conditions (e.g. *Eupatorium cannabinum, Tussilago farfara* and *Ranunculus sceleratus*), although there is no evidence of infection in their natural habitats (Elphinstone, 1996).

In temperate regions, *R. solanacearum* could be detected in field soil for no longer than two years after harvest of potato crops wilted (Shamsuddin et al., 1978), thus suggesting that long term survival and overwintering is more likely linked with infection of perennial reservoir hosts. Infection of these reservoir hosts provides *R. solanacearum* a shelter from cold and harsh environmental conditions while the susceptible crop plant is not available, thus these wild plant species have been suggested to help the pathogen overwinter and provide new inoculum sources for the following cropping season.

1.2.2.1 *S. dulcamara* as a reservoir host of *R. solanacearum*

Amongst the different reservoir hosts of *R. solanacearum*, *S. dulcamara* might be the most frequently identified harbouring the bacterial pathogen. *S. dulcamara*, commonly named bittersweet nightshade, is a perennial climbing shrub woody at the base of the stem with purple flowers and reddish oval berries that are toxic to humans and rabbits, but some birds can digest and spread the seeds around (Gunn & Gaffney, 1974) (Figure 9). *S. dulcamara* is a diploid species (2n = 24), from the subgenus *Potatoe* (Child & Lester, 2001) -also including cultivated potato and its wild relatives- inside the genus *Solanum* (Weese & Bohs, 2007), . *S. dulcamara* is one of the few *Solanaceae* species native from Europe, but it has been recently naturalized to temperate regions in the entire Holarctic area (Horvath et al., 1977) (Figure 10). The natural habitat of this plant species comprises very contrasting environments, from wet habitats of irrigation areas, river and lake shores to dry areas of dunes and plains (T. M. Golas et al., 2010).

Figure 9. Morphological characteristics of *Solanum dulcamara*. Photography by J. Elphinstone, Central Science Laboratory, York, UK, Crown copyright.

Moreover, *S. dulcamara* is frequently found in the vicinity of potato fields, an important factor for participating in the epidemiology of potato-related diseases. For many years now, *S. dulcamara* has been known to be a host of the potato late blight causal agent *Phytophthora infestans* (de Bary, 1876). However, *S. dulcamara* hardly suffers from late blight symptoms, even under very favourable conditions for disease development (Tomek M. Golas et al., 2010). In the last 10 years, two different resistance genes against *P. infestans* were discovered in *S. dulcamara*: *Rpi-dlc 1* and *Rpi-dlc 2*. *Rpi-dlc 1* was the first R-gene discovered in *S. dulcamara* (Tomasz Michal Golas et al., 2010), but the analysis of the genetic map next to the gene suggested that more *Rpi* genes might be present. Confirming this hypothesis, the second *Rpi-dlc 2* resistance gene was discovered shortly after (T. M. Golas, 2010). All these studies concluded that *S. dulcamara* infections with *P. infestans* in nature are very scarce and that the plant does not seem to be an overwintering reservoir, thus not being highly relevant in the late blight epidemiology (Tomek M. Golas et al., 2010). However, the two R-genes found in *S. dulcamara* against *P. infestans* make the bittersweet a good source of resistance genes to cope with potato late blight disease (T. M. Golas, 2010; Tomasz Michal Golas et al., 2010).

For long time now, *S. dulcamara* has been described as one of the reservoir hosts of the bacterial wilt pathogen *R. solanacearum* (Hayward, 1991). *S. dulcamara* plants growing next to potato fields in different locations in Sweden were infected with *R. solanacearum*, providing an overwintering shelter while the bacterium was not detected in surrounding irrigation water (Olsson, 1976). Later on, *S. dulcamara* plants in river banks with their roots submerged in water both in England (Elphinstone et al., 1998) and similarly in the Netherlands (Janse et al., 1998), both reports suggesting a tight control of *S. dulcamara* as they could serve as overwintering shelters and inoculum sources of *R. solanacearum*.

Interestingly, all the cases of identification of *S. dulcamara* harbouring *R. solanacearum*, consisted of the cold-adapted *R. solanacearum* phylotype II-B1 strain (also referred as race 3 biovar 2) (Elphinstone et al., 1998; Janse et al., 1998; Olsson, 1976). Since *R. solanacearum* is not indigenous of Europe, different scenarios are possible origins of these bacterial wilt outbreaks. The first one is that contaminated material were banned for human consumption, but they were for example used for cattle feeding, thus re-entering the agricultural cycle. The second possibility is that waste-treatment plants were not properly cleaning contaminated material and thus *R. solanacearum* was leaking to the environment, where *S. dulcamara* plants could shelter the bacterium during winter and release it for further infections during warm months. The third scenario is that infected breeding material from endemic areas (e.g. South America) was introduced to Europe (Janse, 1996).

The chance that *R. solanacearum* phylotype IIB-1 strains could be permanently established in different European countries a part from the Netherlands where it is already established since the early 90s (Janse et al., 1998), poses at big risk this uniquely quarantine region, thus being crucial to understand how the pathogen interacts with one of its main reservoir hosts in Europe: *S. dulcamara* (Figure 10).

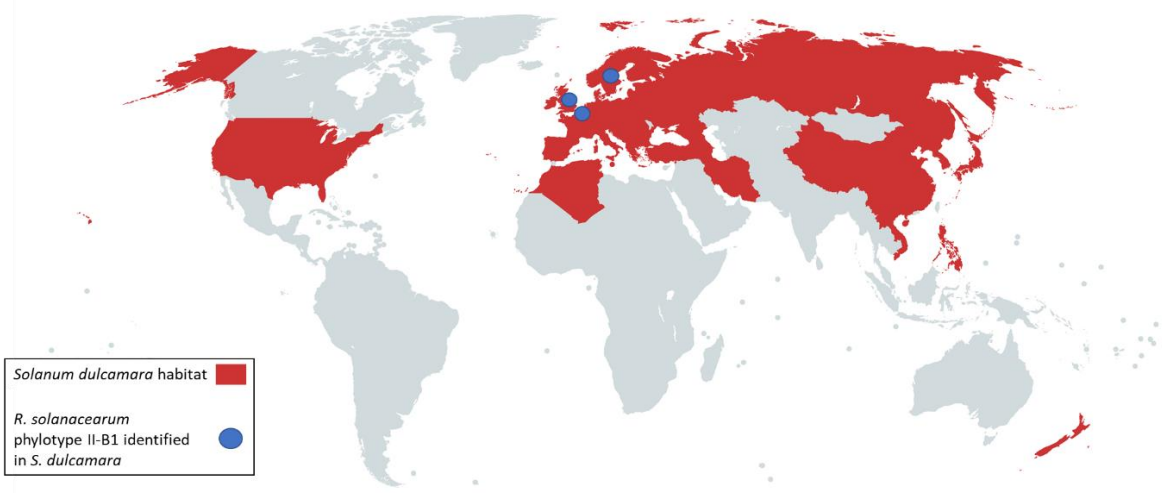


Figure 10. *S. dulcamara* habitat and *R. solanacearum* co-ocurrences. *S. dulcamara* is found in all the holarctic region (red), and several reports have identified cold-adapted *R. solanacearum* phylotype II-B1 strains in different temperate locations (blue circles).

1.3. *RALSTONIA SOLANACEARUM* VIRULENCE DETERMINANTS

Among the vast diversity of bacterial plant pathogens, each of them deploys a different strategy to successfully colonize their plant hosts. Based on the ranking made recently by

Mansfield *et al.* (Mansfield et al., 2012), the infection strategy of the top three bacterial phytopathogens (i.e. *P. syringae*, *R. solanacearum* and *Agrobacterium tumefaciens*) is presented below, focusing on the causal agent of bacterial wilt *R. solanacearum.*

P. syringae spp. is an air-borne pathogen which is spread through air and rain to long distances (Morris et al., 2013). It is a good epiphytic bacterium, which enters plant leaves through natural openings or wounds and stays inside the leaf apoplast within a few millimetres from the entry point (Xin & He, 2013). The most dangerous weapon that *P. syringae* possesses is the Type III Secretion System (T3SS), which injects bacterial effectors inside the plant cell, blocking different defence mechanisms as well as hijacking the plant metabolism to its own benefit (Preston, 2000). *P. syringae* also presents other important virulence factors such as the type IV pili, crucial for the attachment to the leaf surface and UV light protection (Roine et al., 1998). Moreover, *P. syringae* produces the exopolysaccharide alginate that protects the bacterium from oxidative stress in the apoplast (J. Yu et al., 1999). Finally, *P. syringae* produces coronatine, a phytotoxin analogue to jasmonic acid isoleucine responsible for inhibiting salicylic acid accumulation (Elizabeth & Bender, 2007) and the chlorotic area surrounding the necrotic spots in the leaves (Uppalapati et al., 2008). Coronatine has been also shown to suppress stomata closure, thus facilitating the pathogen entry inside the leaf (Melotto et al., 2006). The role of coronatine has been also linked to promoting bacterial growth in the apoplast of different plant hosts (Bender et al., 1987; Brooks et al., 2005; Elizabeth & Bender, 2007).

Agrobacterium tumefaciens is the causal agent of crown gall disease on many different plant hosts (Escobar & Dandekar, 2003). Besides the lethality of the disease it causes, the pathogen is also relevant for the improvements in plant biotechnology and genetic transformation that it facilitated (Zambryski et al., 1983). *A. tumefaciens* is a soil-borne plant pathogen that upon sensing of plant compounds released from wounded plant cells (e.g. phenolic compounds such as acetosyringone, sugars, amino acids) is attracted to the wounded site, where it enters the root system (Gelvin, 2000; Tzfira & Citovsky, 2002; Winans, 1992). The bacterium follows the disease cycle by attaching to the plant cell through a two-step process, first by an acidic polysaccharide, second by cellulose fibrils (Gelvin, 2000). The most interesting point of the *A. tumefaciens* infection strategy involves the delivery of a tumorigenic T-DNA fragment that is inserted into the plant genome through the interaction of plant proteins (Van Attikum et al., 2001; Ziemienowicz et al., 2000). Once the T-DNA is inserted in the plant genome, two different types of genes are expressed: oncogenes and opine-related genes. The oncogenes promote auxin and cytokinin production at the infected cell, which finally leads to tumour

formation (Zhu et al., 2000). The opine-related genes are involved in the plant production of amino acid and sugar phosphate derivatives called opines, which can be catabolized by *A. tumefaciens,* maintaining the bacterial population (Desseaux, 1993).

R. solanacearum has some similarities to the strategies of *P. syringae* and *A. tumefaciens* above described. Like *A. tumefaciens, R.* solanacearum is also a soil-borne plant pathogen, so it senses the roots and enters through wounds or secondary roots (J. Vasse et al., 1995; Yao & Allen, 2006). Like *P. syringae,* the main virulence factor of *R. solanacearum* is also the T3SS and the effectors that injects in the plant cell (Boucher et al., 1985). For the scope of this thesis, all the virulence factors of *R. solanacearum* will be described in detail below.

1.3.1 The Type III Secretion System and the Type III Effectors

By 1985, a large random mutagenesis study to identify virulence-related genes in *R. solanacearum* was able to identify the *hrp* (hypersensitive response and pathogenicity) cluster (Boucher et al., 1985), simultaneously reported also in *P. syringae* (Lindgren et al., 1986) and homologous with the Yop and Ipa secretion systems in *Yersinia pestis* and *Shigella flexneri* (Gough et al., 1992). The *hrp* cluster contains more than 20 genes, transcriptionally regulated by the master regulators HrpB and HrpG (Brito et al., 1999; Genin et al., 2005). *R. solanacearum* mutants lacking any of the *hrp* genes are completely avirulent and unable to elicit HR, hence the T3SS is considered *R. solanacearum* main virulence factor (Boucher et al., 1987), as well as of many other bacterial phytopathogens (Table 2).

The T3SS apparatus is not the virulence factor *per se*, but because of the associated type III effectors (T3Es) that are injected in the plant cell with a variety of functions (Macho & Zipfel, 2015). *R. solanacearum* species complex contain the largest set of T3Es compared to other bacterial phytopathogens (Genin & Denny, 2012), with approximately more than 100 different T3Es (Peeters, Carrère, et al., 2013). Based on protein domain similarity, many *R. solanacearum* T3Es are part of multimember families: *PopA/B/C*, *AWR2/3/4/5_1/5_2*, *SKWP1/2/3/5/7*, *HLK1/2/3* and *GALA2/3/4/5/6/7* (Poueymiro & Genin, 2009). Even though the biochemical activity of most T3Es still remains unknown, during the last years, genetic and biochemical studies have shed some light on this topic. For instance, the *GALA* effectors (e.g. *ripG2, ripG3, ripG4*) contain an F-box domain that targets host cell proteins to the proteasome and these effectors are required for full bacterial virulence (Remigi et al., 2011). The *AWR* effectors (e.g. *ripA2, ripA3, ripA5*) have been shown to specify virulence or avirulence depending on the plant host (Solé et al., 2012). Although in most of the single T3Es deletion mutants no loss-of-virulence phenotype was observed, some effector mutants proved to affect

virulence: *ripA2* (AWR2), *ripD* (brg8) (Cunnac et al., 2004), *ripF1_1* (PopF1) (Meyer et al., 2006) and *ripR* (PopS) (Jacobs et al., 2013). Accordingly, a recently published study analysing the effect of multiple *R. solanacearum* T3E deletions demonstrated that the simultaneous deletion of 42 T3Es was required to observe a major compromise on virulence and xylem proliferation in tobacco and eggplant (Lei et al., 2020). Due to the T3Es redundant function in pathogenicity, HR elicitation or toxic effects to the host, the biological function of most T3Es is still poorly understood (Coll & Valls, 2013).

1.3.2 Type II Secretion System and Cell Wall Degrading Enzymes

The second most important virulence secretion system of *R. solanacearum* is the Type II Secretion System (T2SS), encoded by the *sec* and *gsp* clusters (Table 2). Its crucial role in virulence was demonstrated when the T2SS-deficient mutant had a notable decrease in virulence in tomato (Kang Yaowei et al., 1994). A proteomic study identified a total of 36 proteins secreted through the T2SS (Zuleta, n.d.), six of them functioning as cell wall degrading enzymes (CWDE), with important individual contributions to virulence: the polygalacturonases PglA, PehB and PehC (E. T. González & Allen, 2003; Q. Huang & Allen, 1997; M. A. Schell et al., 1988), the Egl endoglucanase (D. P. Roberts et al., 1988), the Pme pectin methylesterase (Tans-Kersten et al., 1998) and the CbhA cellobiohydrolase (Huanli Liu et al., 2005). The remaining 30 T2 secreted proteins might have a role in virulence, although still unknown, since the mutant lacking the 6 CWDE genes was still more virulent than the complete T2SS mutant (Huanli Liu et al., 2005).

1.3.3 Type V and VI Secretion Systems

The Type V Secretion System (Tat-secretory pathway; T5SS) was shown to be involved in tomato virulence (E. T. González et al., 2007), but its secretome includes genes involved in different bacterial activities such as polygalacturonase activity, ability to metabolize nitrate, *in planta* growth, which explains the pleiotropic effect of the T5SS mutant (E. T. González et al., 2007) (Table 2).

The Type VI secretion system (T6SS) is the latest of the secretion systems to be identified in *R. solanacearum*, from a search of T6SS orthologous genes in other bacteria (Shrivastava & Mande, 2008) (Table 2). T6SS mutants were shown to affect the secretion of a total of 38 proteins (Zhang et al., 2012), to reduce bacterial virulence when soil-inoculated (Zhang et al., 2012) and linked the T6SS with biofilm formation and motility (Zhang et al., 2012, 2014).

1.3.4 Exopolysaccharide secretion

The main responsible for xylem clogging is the massive production of exopolysaccharide (EPS), which is an acidic secreted polymer composed of three major units: N-acetylgalatosamine, 2-N-acetyl-2-detoxy-galacturonic and 2-N-acetyl-4-N-(3-hydroxybutanoyl)-2,4,6-tri-deoxy-D-glucose (Orgambide et al., 1991) (Table 2). The enzymes that synthesize and export this compound are encoded in the *eps* operon with 7 genes (*epsAPBCDEF*) (Jianzhong Huang & Schell, 1995), under the same promoter, activated at bacterial densities above 10^7 CFU/ml (Steven J. Clough, Flavier, et al., 1997; Garg et al., 2000; J. Huang et al., 1995). Although it was firstly suggested that *eps* mutants would be completely avirulent (Kelman, 1954), later on it was shown that these *eps*-deletion mutants can cause delayed wilting symptoms in tomato inoculated plants (Kao et al., 1992; Saile et al., 1997). EPS localization showed that although around 80% of the EPS is secreted to the environment and 20% is kept cell-bound (McGarvey et al., 1998). The secreted EPS is the responsible for xylem clogging and vascular cell rupture causing the wilting symptoms, while the cell-bound EPS is suggested to provide a bacterial capsule to mask MAMPS from plant defence receptors (McGarvey et al., 1998; Mark A. Schell, 2000).

1.3.5 Biofilm formation

Bacterial biofilms protect bacteria from different kinds of stresses such as desiccation, antibiotics, and host antimicrobial defences and help them attach, feed and dispose of waste in fluid environments (Danhorn & Fuqua, 2007; Flemming & Wingender, 2010; Morris & Monier, 2003). Biofilms vary in composition, but are generally composed of live and dead cells, embedded in a matrix of polysaccharides, proteins and DNA (Montanaro et al., 2011). In *R. solanacearum*, biofilms are of extreme importance in adapting to the fluid xylem environment as well as allowing the bacteria to spread throughout the vasculature (Tran et al., 2016). A recent study showed that *R. solanacearum* mutants lacking two extracellular DNases (exDNases) formed non-spreading colonies and abnormally thick biofilm *in vitro*, as well as reduced bacterial spread in tomato xylem vessels and decreased virulence *in planta* (Tran et al., 2016). *In vitro* studies showed that *R. solanacearum* biofilm formation depends on different factors such as aerotaxis, the mannose-binding lectin LecM, twitching and swimming motility and EPS (Corral et al., 2020; Kang et al., 2002a; Meng et al., 2011, 2015; Saile et al., 1997; Yao & Allen, 2006).

1.3.6 Host attachment and bacterial motility

As a soil-borne and vascular pathogen, *R. solanacearum* needs a good ability to attach not just to the roots but also to the xylem vessels of the plants host. In *R. solanacearum*, host attachment is mediated by bacterial lectins and by pili. Lectins are highly specific carbohydrate-binding proteins that confer host recognition and binding (Romantschuk, 1992) (Table 2). In *R. solanacearum,* at least three types of lectins have been identified with different binding specificities: LecM, LecX, and LecF. LecM, which transcription is activated by HrpG (Valls et al., 2006), was reported to have a role in biofilm formation and virulence in tomato plants (Mori et al., 2015). In addition to lectins, type IV pili, which are filamentous appendages that protrude outside bacterial cells, have been also shown to participate in *R. solanacearum* attachment, biofilm formation and virulence (Wairuri et al., 2012).

To successfully colonize different plant tissues, *R. solanacearum* needs to coordinate specific types of motility such as swimming (individual movement in liquid or semisolid medium), and twitching (individual movement on solid surfaces) (Corral et al., 2020) (Table 2). Swimming is mediated by rotating polar flagella, and mutants lacking either FliC (the flagellar subunit protein) or FliM (the flagellar motor switch protein) are nonmotile and present reduced virulence on soil-soaked tomato plants (Tans-kersten et al., 2001). *R. solanacearum* can direct the movement towards specific chemical gradients through chemotaxis. Cell membrane-associated receptors called MCPs (methyl-accepting chemotaxis proteins), detect environmental stimuli and change their conformation triggering autophosphorylation of CheA, which forms a complex with CheW, that finally interacts with the flagellar motor (FliM), changing its direction of rotation. *CheA* and *cheW R. solanacearum* mutants are motile but nonchemotactic, and their virulence is as low as that of a completely nonmotile *fliC* knockout mutant (Yao & Allen, 2006).

Twitching motility is driven by extension, attachment, and retraction of the type IV pilus in solid surfaces or solid media. In *R. solanacearum*, type IV pili have been implicated in natural transformation, biofilm formation, and virulence (Kang et al., 2002a) (Table 2). Inactivation of PilA, PIlQ or PilT (monomers of the major pilin protein, the secretin involved in pilus extrusion and the protein required for pilus retraction, respectively), reduces both twitching motility and virulence (Kang et al., 2002a; H. Liu et al., 2001). A recent study from our team investigated the role of pilus-mediated chemotaxis showing that *pilI* (encoding a CheW-like protein) and *chpA* (coding CheA-like protein) mutants had altered biofilm formation, reduced twitching motility, transformation efficiency and root attachment. In addition, these two mutants only displayed impaired virulence on tomato plants when soil-inoculated (Corral et al., 2020).

1.3.7 Metabolic adaptation and phytohormone production

During plant colonization, *R. solanacearum* differentially activates or represses specific metabolic pathways that allow a modulated adaptation to the different environments encountered in the plant. One of the first *in planta* transcriptomic studies of *R. solanacearum* discovered that sucrose uptake and catabolism genes were highly upregulated during *in planta* colonization (Jacobs et al., 2012). The *scrA* mutant, lacking the gene that encodes for the enzyme that converts sucrose to sucrose-6-phosphate, had reduced virulence in tomato, potato and *S. dulcamara* plants, indicating the importance of sucrose metabolism for a proper pathogenesis (Jacobs et al., 2012) (Table 2). During xylem colonization, *R. solanacearum* faces a hypoxic environment, which could limit its growth. However, a recent study showed that the nitrate concentration in the xylem vessels is optimal for *R. solanacearum* growth (Dalsing et al., 2015). *R. solanacearum* genome encodes for the necessary enzymes to use nitrate as an energy source (i.e. dissimilatory nitrate reduction), to incorporate nitrate as a molecular building block (i.e. assimilatory nitrate reduction) (Dalsing & Allen, 2014) and to detoxify reactive nitrogen species (i.e. denitrification) (Dalsing et al., 2015) (Table 2). The ability to use nitrate as terminal electron acceptor has been proposed to sustain rapid bacterial growth in the xylem (Dalsing et al., 2015). Another transcriptomic study investigating the genes regulated by EfpR in an evolutionary context *in planta*, found that multiple metabolic pathways such as amino acid, organic acid, genes involved in secondary metabolism, and fatty acid biosynthesis are directly or indirectly regulated by EfpR (Perrier et al., 2016) (Table 2).

R. solanacearum secondary metabolite ralfuranone was also shown to play an important role in pathogenicity. The *ralA* mutant, lacking the furanone synthase, had reduced virulence in tomato plants (Kai et al., 2014) (Table 2). Another example of secondary metabolite involved in *R. solanacearum* virulence is putrescine, which was accumulated in xylem sap during infection and a *R. solanacearum speC* mutant, responsible for putrescine biosynthesis, caused complete loss of virulence in tomato infection (Lowe-Power, Hendrich, et al., 2018) (Table 2).

Interestingly, *R. solanacearum* genome codes for phytohormone biosynthetic genes to produce auxin (Valls et al., 2006), cytokinin (Genin & Denny, 2012) and ethylene (Freebairn & Buddenhagen, 1964) (Table 2). Although their precise function is still unknown, ethylene has been involved in virulence during *A. thaliana* infection (Hirsch et al., 2002).

1.3.8 Protective enzymes and efflux transporters

Upon bacterial infection, plant defence mechanisms start producing high amounts of reactive oxygen species (ROS), plant-derived phenolic compounds and other antimicrobials. *R. solanacearum* encodes for ROS detoxification enzymes such as polyphenol oxidases (Hernández-Romero et al., 2005), catalases (i.e. *katE, katG*), peroxidases (*bcp*), superoxide dismutases (*sodBC*) and alkyl hydroperoxide reductases (i.e. *ahpC1/C2/D/F*) (Flores-Cruz & Allen, 2009; Valls et al., 2006), which expression is under the regulation of OxyR (Flores-Cruz & Allen, 2011) (Table 2). Although no clear link between *ahp* genes and *R. solanacearum* virulence, several studies have shown the induction of *ahp* genes in biofilm-forming cells in different bacterial pathogens, contributing to bacterial epiphytic survival and attachment in the intercellular spaces or to the xylem vessels (Büttner & Bonas, 2010a; Jang et al., 2016; Oh & Jeon, 2014; Panmanee & Hassett, 2009; Wasim et al., 2009). A slight decrease in virulence was shown in a *bcp*, the *dps* iron-binding oxidoreductase and the *oxyR* mutants, proving their role in *R. solanacearum* pathogenicity (Flores-Cruz & Allen, 2009).

R. solanacearum can also survive to the antimicrobial compounds secreted by plant cells upon infection by extruding toxins through membrane-bound efflux pumps, commonly named multidrug resistance efflux pumps (MDRs). Two *R. solanacearum* MDRs were identified to be induced during plant colonization: *acrA* and *dinF* (Brown et al., 2007) (Table 2). *AcrA* and *dinF* were also upregulated by toxic and DNA-damaging compounds and carbon limitation. *R. solanacearum acrA* and *dinF* deletion mutants had reduced virulence on tomato and decreased the ability of the bacterium to grow in the presence of antibiotics, phytoalexins and detergents (Brown et al., 2007).

Table 2. Main virulence factors in *R. solanacearum*. Adapted from Genin 2010

Function	Genes/Products	Role in pathogenesis	Reference
T3SS & T3Es	*hrp/hrc* genes (T3SS)	T3 secretion apparatus	(Boucher et al., 1985, 1987)
	T3Es	Host specificity, suppression host defenses	(Peeters, Carrère, et al., 2013)
	hpa genes	T3E folding and translocation	(Lonjon et al., 2016)
T2SS & CWDE	*gsp* genes (T2SS)	T2 Secretion apparatus	(Kang Yaowei et al., 1994)
	egl, pme, pglA, pehBC, cbhA (CWDE)	Plant cell wall degradation	(Huanli Liu et al., 2005)
T5SS & T6SS	*tat* gene cluster (T5SS)	Role in virulence	(E. T. González et al., 2007)
	tss genes (T6SS)	Mutants with reduced virulence, motility and biofilm	(Zhang et al., 2012)
EPS	*eps* gene operon	Plant vasculature clogging	(T. P. and S.-R. B. Denny, 1991)
ROS scavenging enzymes	catalases, peroxidases, superoxide dismutases	ROS detoxification	(Flores-Cruz & Allen, 2009)
Attachment & Motility	Lectins	Adherence to plant cells	(Romantschuk, 1992)
	Flagella	Swimming	(Tans-kersten et al., 2001)
	Type IV pili	Twitching and attachment	(Kang et al., 2002a)
	chemotaxis	Host specificity	(Yao & Allen, 2006)
Efflux	*acrA, dinF*	Resistance to antimicrobials	(Brown et al., 2007)
Metabolic parasitism	Nitrogen metabolism	Metabolic versatility *in planta*	(Dalsing et al., 2015)
	Sucrose catabolism	Metabolic versatility *in planta*	(Jacobs et al., 2012)
	Ralfuranones	Mutants with reduced virulence	(Kai et al., 2014)
	EfpR	Mutants with reduced virulence	(Perrier et al., 2016)
	Putrescine	Mutants with reduced virulence	(Lowe-Power, Khokhani, et al., 2018)
Phytohormones	Auxin, cytokinin and ethylene biosynthesis	Role unknown	(Valls et al., 2006)

1.4 *R. SOLANACEARUM* GENE EXPRESSION ANALYSES

The sequence of *R. solanacearum* strain GMI1000 genome in 2002 represented a big advance in understanding and identifying many virulence genes and their regulation (Salanoubat et al., 2002). Analysis of the genome showed that it has a bipartite structure with a chromosome (~3.7Mb) and a megaplasmid (~2.1Mb). *R. solanacearum* genome has a high proportion of G+C in most of the genome except for the pathogenic islands disseminated through horizontal gene transfer (Genin & Denny, 2012).

R. solanacearum species complex is defined by its wide range of host specificities, aggressiveness or optimal growth temperatures (Prior & Fegan, 2005). To understand this variability, genomes of different *R. solanacearum* strains became available (Chen et al., 2017; Guarischi-Sousa et al., 2016; Hayes et al., 2017; Li et al., 2011; Patil et al., 2017; Remenant et al., 2010, 2012; Sun et al., 2017; Xu et al., 2011). A representative sample of 19 genomes from the four different *R. solanacearum* phylotypes was used to identify candidate host-specific genes, such as T3Es associated with specific strains (Ailloud et al., 2015).

In the following section, the main genetic regulation networks in *R. solanacearum* to modulate the expression of the virulence factors and *in planta* fitness are described, finishing with the last high-throughput studies and the need for expanding our understanding of *R. solanacearum* to *in planta* conditions.

1.4.1 The *phc* sensing system

The *phc* network is one of the main regulatory systems in *R. solanacearum* and PhcA is its central regulator. PhcA was discovered as the responsible of the Phenotype Conversion, which consists in a loss of virulence together with decrease of EPS and endoglucanase (Egl) production, while it activates motility, siderophore biosynthesis and endo-polygalacturonase (PglA) (Genin & Boucher, 2002) (Figure 11). Based on *in vitro* studies, it was suggested that a specific volatile compound produced by *phcB* should control the activity of PhcA (S. J. Clough et al., 1994). The volatile compounds responsible for activating PhcA expression at high cell densities surpassing its repression from the Phcs/PhcR two-component system, were 3-hydroxypalmitic acid methyl ester (3-OH-PAME) and methyl 3-hydroxymyristate (3-OH-MAME) (Steven J. Clough, Lee, et al., 1997; Flavier, Clough, et al., 1997). Other two-component systems were identified to activate EPS expression, such as the VsrB/VsrC and XpsR, the latter's transcription being regulated by VsrA/VsrD and PhcA (J. Huang et al., 1993, 1995) (Figure 11).

As a comprehensive summary of the *phc* system, it acts as a master switch of different virulence genes in a cell density-dependent manner. At cell densities below 10^7 cells/ml, 3-OH-PAME is low and PhcA active, inducing bacterial motility. At cell densities above 10^7 cells/ml, 3-OH-PAME accumulates, PhcA is induced by PhcS/PhcR, activating the expression of the EPS operon and PglA (Figure 11).

1.4.2 The *SolR/SolI* quorum sensing system

R. solanacearum possess the SolI/SolR system, analogue to the *phc* system in the sense that is auto-induced by a cell density-dependent manner. SolI is the enzyme involved in the synthesis of the autoinducer molecule called N-hexanoyl and N-octanoyl-homoserine lactones, which accumulation leads to SolR activation, further inducing the expression of *aidA* (Flavier, Ganova-Raeva, et al., 1997) (Figure 11). In addition to that, the SolI/SolR system is also induced by 3-OH-PAME accumulation via PhcA (Flavier, Ganova-Raeva, et al., 1997). Control of SolR/SolI by the *phc* system gives *R. solanacearum* a two-stage hierarchical cell density sensing system. First, the *phc* system targets are not activated until density exceeds $\sim 10^7$ cells/ml. Second, SolI/SolR-regulated targets are only activated when density exceeds $\sim 10^8$ cells/ml (Mark A. Schell, 2000). It is likely that the SolI/SolR quorum system activates genes that have a role in the terminal stages of the infection. Consistent with this idea, SolI/SolR expression is regulated by the alternate sigma factor RpoS, which in many bacteria is associated with stationary phase survival and environmental stress (Flavier et al., 1998) (Figure 11).

1.4.3 The T3SS regulatory cascade

As in many other gram-negative bacteria, the main virulence factor of *R. solanacearum* is the T3SS, encoded in the *hrp* (hypersensitive response and pathogenicity) and the *hrc* (hypersensitive response and conserved) cluster (Salanoubat et al., 2002). *R. solanacearum hrp/hrc* cluster contains more than 20 genes organized in seven transcriptional units (Arlat et al., 1992; van Gijsegem et al., 1995). Apart from including the T3SS genes, the *hrp* cluster also comprises T3Es and *hpa* (HR and pathogenicity associated) (Salanoubat et al., 2002) (Figure 11). The first expression analysis of the *hrp* cluster in artificial media revealed that it is activated in minimal medium while repressed in rich medium (Arlat et al., 1992). Upstream *hrp* regulators were only discovered after plant cell co-cultures, identifying PrhA as the plant signal receptor (Marenda et al., 1998), which sequentially activates PrhR/PrhI, PrhJ and finally HrpG that induces the expression of HrpB (Brito et al., 1999, 2002) (Figure 11).

HrpG and HrpB are two master regulators of virulence in *R. solanacearum*. On one hand, HrpG regulates a HrpB-independent pathway with genes related to cell-wall degradation (*pehB, egl*),

ROS scavenging (*katE*), EPS regulation (*epsR*), and auxin and ethylene production (*efe*) (Valls et al., 2006) (Figure 11). On the other side, HrpG transcriptionally activates HrpB, which induces T3SS structural genes (encoded by *hrp* and *hrc* clusters) and T3Es (Cunnac et al., 2004) (Figure 11). *hrpG* mutants are defective in crossing the root endodermis and reach the vasculature, and *hrpB* mutants have lower multiplication and colonization ability in xylem vessels (Jacques Vasse et al., 2000). Recently, the role of several *hpa* genes has been elucidated as post-transcriptional regulators of T3E translocation (Lonjon et al., 2016). HpaB, HpaD are chaperones that ensure a proper T3E folding for a coordinated translocation through the T3SS (Parsot et al., 2003; Thomas et al., 2012). HpaP was recently shown to repress the secretion of HrpY pillin and AvrA effectors in early infection, while acting as a helper for PopP1 secretion (Lohou et al., 2014). HpaG (also known as LrpE) is a leucine-rich repeats containing protein that negatively regulates the production of Hrp pili to disperse bacterial cells from aggregates, thus facilitating bacterial movement in the xylem vessels (Murata et al., 2006). Survival experiments on *hpa* mutants showed that *hpaB* mutant has impaired virulence on both *A. thaliana* and *Medicago truncatula*, while *hpaG* mutant is only less virulent on *M. truncatula* (Lonjon et al., 2016).

Finally, the *hrp* cluster is also transcriptionally repressed by PhcA by inactivating the *prhI/prhR* promoter in rich medium (Yoshimochi et al., 2009), indicating the interconnection of *R. solanacearum* signalling pathways integrated at different levels.

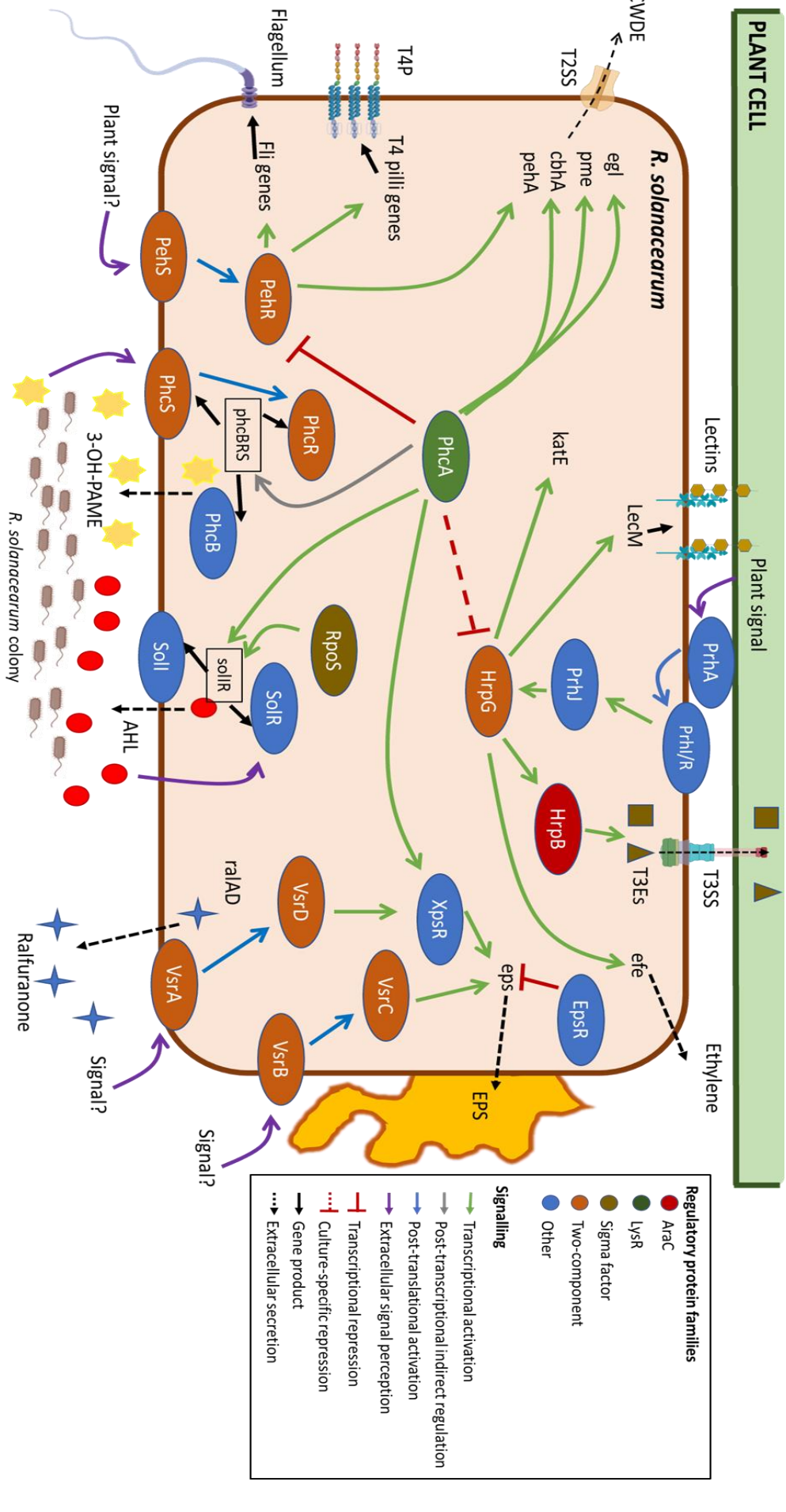


Figure 11. Scheme of the *phc*, *sol* and *hrp* regulatory cascades and associated virulence factors in *R. solanacearum*.

1.5 THE OMICS ERA IN *R. SOLANACEARUM*: FROM *IN VITRO* TO *IN PLANTA* STUDIES

The use of *in vitro* conditions was the first big step in the identification of novel virulence factors in *R. solanacearum* such as the EPS (J. Huang et al., 1995), the HrpG and HrpB regulons (Occhialini et al., 2005; Valls et al., 2006), adhesins and biofilm formation (Saile et al., 1997), and specific components of the *phc* system (S. J. Clough et al., 1994). Although minimal medium was used to mimic the plant environment, many *R. solanacearum* genes specifically involved in host adaptation or interaction with plant cells could only be detected using *in planta* assays.

The first *R. solanacearum in planta* studies used the In Vivo Expression Technology (IVET), which consists of a library of bacterial promoters cloned upstream of a promoterless copy of an *in planta* growth-required gene and introduced in a bacterial strain lacking this gene (Rainey & Preston, 2000). Different *R. solanacearum* mutant strains were inoculated into tomato plants and only those mutant strains whose promoters were upregulated *in planta* were recovered during the infection (Brown & Allen, 2004). Among the identified *R. solanacearum* genes which were involved in transport, metabolism and stress response, as well as previously described virulence genes (e.g. *vsrB, vsrD, rpoS*), and only 60% overlapped with *R. solanacearum* genes induced in minimal medium (Brown & Allen, 2004). However, since the IVET technology only allowed the identification of highly expressed genes, transcriptomic studies started to appear. The first global gene expression analysis of *R. solanacearum in planta* was performed in 2012 by Jacobs *et al.* (Jacobs et al., 2012) in tomato plants at the onset of the disease, underscoring the importance of sucrose, and nitrate assimilation and respiration during tomato xylem colonization (Jacobs et al., 2012). The following transcriptomic studies investigated specific genes induced upon infection of different hosts (Ailloud et al., 2016), the genes that allowed cold adaptation to phylotype II-B1 strains (Meng et al., 2015), the bacterial genes differentially expressed in the roots of the resistant potato wild relative *Solanum commersonii* (Puigvert et al., 2017) and the whole regulon under the control of PhcA (Khokhani et al., 2017).

Summarizing, these transcriptomic reports have confirmed that *R. solanacearum* modulates its gene expression depending on different factors such as bacterial growth, host specificity, or temperature. The importance of *in planta* studies was highlighted when it was shown that the T3SS are expressed at high bacterial densities at late infection stages *in planta*, contrary to what *in vitro* experiments had proven. Finally, *R. solanacearum* has a complex infection cycle, colonizing different plant tissues from the root apoplast to the massively clogged xylem vessel

and the surrounding parenchyma cells at late stages. To date, *R. solanacearum* gene expression studies have mostly focused on the disease onset stage. Therefore, it is of utmost need to tackle all the different environments that *R. solanacearum* colonizes in the plant in order to obtain a dynamic and complete picture of its infection process to be able to defeat the pathogen.

OBJECTIVES

The main goals of this thesis are described below:

Characterization of the dynamic transcriptional profile of *Ralstonia solanacearum* in different infection stages in potato plants

1. To identify the dynamic gene expression pattern of key virulence factors of *R. solanacearum* through the infection process in potato plants.

2. To decipher if *R. solanacearum* differentially adapts to the main plant environments by deploying specific gene sets.

Characterization of *Solanum dulcamara* as a reservoir host of *Ralstonia solanacearum*

4. To characterize the symptomatology developed over time by *S. dulcamara* in response to *R. solanacearum* compared to a susceptible tomato host (*cv* Marmande*).*

5. To characterize the tissue localization of *R. solanacearum* during the colonization of *S. dulcamara*.

6. To identify possible mechanisms that enable *S. dulcamara* to tolerate *R. solanacearum* infections.

7. To test the role of *S. dulcamara* as an overwintering host of *R. solanacearum.*

CHAPTER 1:

Dynamic expression of *Ralstonia solanacearum* virulence factors and metabolism-controlling genes during plant infection

Dynamic expression of *Ralstonia solanacearum* virulence factors and metabolism-controlling genes during plant infection

R. de Pedro-Jové[1,2¥], M. Puigvert[1,2¥], P. Sebastià[2¥], A.P. Macho[3], J. S. Monteiro[4], N. S. Coll[2], J.C. Setubal[4], M. Valls[1,2*]

[1] Department of Genetics, University of Barcelona, Barcelona, Catalonia, Spain
[2] Centre for Research in Agricultural Genomics (CSIC-IRTA-UAB-UB), Bellaterra, Catalonia, Spain
[3] Shanghai Center for Plant Stress Biology, CAS Center for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai 201602, China
[4] Departamento de Bioquímica, Universidade de São Paulo, São Paulo, Brazil

¥Both authors contributed equally to this work

*Correspondence: marcvalls@ub.edu

Abstract

Ralstonia solanacearum is the causal agent of bacterial wilt, a devastating plant disease responsible for serious economic losses especially on potato, tomato, and other solanaceous plant species in temperate countries. In *R. solanacearum*, gene expression analysis has been key to unravel many virulence determinants as well as their regulatory networks. However, most of these assays have been performed using either bacteria grown in minimal medium or *in planta*, after symptom onset, which occurs at late stages of colonization. Thus, little is known about the genetic program that coordinates virulence gene expression and metabolic adaptation along the different stages of plant infection by *R. solanacearum*. We performed an RNA-sequencing analysis of the transcriptome of bacteria recovered from potato apoplast and from the xylem of asymptomatic or wilted potato plants, which correspond to three different infection stages (Apoplast, Early and Late xylem). Our results show dynamic expression of metabolism-controlling genes and virulence factors during parasitic growth inside the plant. Flagellar motility genes were especially up-regulated in the apoplast and twitching motility genes showed a more sustained expression over time. Xylem-induced genes included virulence genes, such as the type III secretion system (T3SS) and most of its related effectors and nitrogen utilisation genes. The upstream regulators of the T3SS were exclusively up-regulated in the apoplast, preceding the induction of their downstream targets. Finally, a large subset of genes involved in central metabolism was exclusively down-regulated in the xylem at late infection stages. This is the first report describing *R. solanacearum* dynamic transcriptional

changes within the plant during infection. Our data define four main genetic programmes that define gene pathogen physiology during plant colonisation. The described stage-specific expression of virulence genes provides key information on the *R. solanacearum* potato infection process.

Key words

Ralstonia solanacearum, Bacterial wilt, RNAseq, Virulence factors, Dynamic Gene Expression, Metabolism, T3SS, effectors, Xylem, Apoplast.

Introduction

Brown rot or bacterial wilt of potato is a vascular disease caused by the bacterial phytopathogen *Ralstonia solanacearum*. This gram-negative β-proteobacterium is among the most threatening bacterial phytopathogens worldwide, as it can infect over 200 different plant species, including many important crops such as potato, tomato, peanut, eggplant and banana (Coll & Valls, 2013; Hayward, 1991; Peeters, Guidot, et al., 2013). Although *R. solanacearum* is endemic of tropical and sub-tropical regions, phylotype IIB-1 strains such as UY031 are acclimated to lower temperatures and have caused important outbreaks in temperate areas (Champoiseau et al., 2009; Ciampi et al., 1980; Janse et al., 2004).

R. solanacearum has a complex life cycle. The pathogen survives in soil and water for long periods of time (Belén Álvarez et al., 2007). When *R. solanacearum* senses the roots of natural hosts by plant exudates (Yao & Allen, 2006), it penetrates the host through the root elongation zone, root wounds or secondary root emerging points (J. Vasse et al., 1995). The root intercellular spaces (the apoplast) constitutes a front line in the arms race in plant-pathogen interactions and it is thus a hostile environment to phytopathogens (Du et al., 2015). Therefore, colonisation of the apoplast is key for *R. solanacearum* pathogenicity (Hikichi et al., 2007; Planas-Marquès et al., 2018; Zuluaga et al., 2013). Successful infections involve entry into the vascular cylinder and extensive colonisation of the xylem vessels (Jacobs et al., 2012; Planas-Marquès et al., 2020). Occlusion of the vasculature due to massive exopolysaccharide (EPS) production and bacterial multiplication ultimately cause wilting symptoms and plant death (Genin, 2010; J. Vasse et al., 1995).

To progress across the different plant tissues, *R. solanacearum* uses a panoply of virulence determinants (Lu et al., 2018; Jacques Vasse et al., 2000). The main virulence factor in this and many other pathogenic bacteria is the Type III Secretion System (T3SS) (Büttner & He, 2009; Coburn et al., 2007), which delivers effector proteins inside the plant cells, hijacking the cellular machinery for bacterial benefit (Peeters, Carrère, et al., 2013). Another key virulence determinant is EPS. EPS leads to the clogging of the xylem vessels and plant symptom appearance, and it can also bind to the cell wall and protect the bacterium from plant defences (J. Huang et al., 1993; Prior P. et al., 2013). In addition, the general secretion system (type II) secretes important virulence factors into the apoplast, including cell wall degrading enzymes (Kang Yaowei et al., 1994). These enzymes are collectively important for *R. solanacearum* plant colonisation, since multiple deletion of the *egl*, *pehA/B/C, pme* and *cbhA* genes compromised pathogenicity (Huanli Liu et al., 2005). Bacterial motility also plays important roles during parasitic life *in planta*. For instance, *R. solanacearum* flagellar components were shown to be

essential at early stages of infection (Tans-Kersten et al., 2004) and mutants in the main twitching gene *pilA* were less pathogenic (Kang et al., 2002b). On the other hand, the *R. solanacearum* genome encodes the necessary enzymes to use nitrate as an energy source (i.e. dissimilatory nitrate reduction), to incorporate nitrate as a molecular building block (i.e. assimilatory nitrate reduction) (Dalsing & Allen, 2014) and to detoxify reactive nitrogen species (i.e. denitrification) (Dalsing et al., 2015). The ability to use nitrate as terminal electron acceptor has been proposed to sustain rapid bacterial growth in the xylem, a hypoxic environment that is nonetheless rich in nitrate (Dalsing et al., 2015; Pegg, 1985).

Gene regulation analyses are essential to decipher how *R. solanacearum* finely tunes its pathogenicity. For instance, transcription of the *hrp* genes -encoding the T3SS- and its related effectors was found to be controlled by the HrpB transcriptional activator. HrpB lies downstream of a regulatory cascade induced by bacterial contact with the plant cell wall (Brito et al., 1999) . The cascade includes the membrane receptor PrhA, the signal transducer PrhI and the transcriptional regulators PrhJ and HrpG, the latter directly activating *hrpB* transcription (Genin et al., 2005; Valls et al., 2006). Gene expression studies demonstrated that the *R. solanacearum hrp* genes and T3SS effectors were transcribed *in planta* at late infection stages (Monteiro, Genin, et al., 2012). Based on these results, it was speculated that *R. solanacearum* could inject T3Es to the xylem parenchyma cells in order to hijack plant defences and manipulate the host metabolism (Monteiro, Genin, et al., 2012). These findings were later confirmed in gene expression studies using bacteria extracted from infected tomato and banana plants (Ailloud et al., 2016; Jacobs et al., 2012) or bacterial transcripts isolated from infected potato roots (Puigvert et al., 2017). Similar to the T3SS, EPS production is also stringently controlled through the expression of the *eps* operon, which encodes all EPS biosynthesis genes (Garg et al., 2000). The *eps* operon promoter is dependent on the global regulator PhcA, whose production is induced at bacterial densities above 10^7 CFU/ml (Steven J. Clough, Flavier, et al., 1997; Garg et al., 2000; J. Huang et al., 1995) . Finally, it has been described that some crosstalk exists between the *eps* and the *hrp* gene regulation, since *hrpG* is negatively regulated by *phcA* (Genin et al., 2005; Yoshimochi et al., 2009).

Bacterial interactions in plant hosts do not consist on one static phase, but rather in a dynamic interaction during disease development. However, all *R. solanacearum in planta* transcriptomic studies have focused so far on a specific stage of the infection process: xylem colonisation at the onset of disease symptoms (Ailloud et al., 2016; Jacobs et al., 2012; Khokhani et al., 2017; Meng et al., 2015), with the exception of a single study indirectly analysing bacterial reads from infected roots (Puigvert et al., 2017). Among the differentially

expressed (DE) genes identified in these previous studies, the T3SS, T3Es, motility genes, ROS detoxifying enzymes and cell wall degrading enzymes were found up-regulated in most cases. Dynamic transcriptomic studies of the model plant pathogen *Pseudomonas syringae* analysing different moments of the disease development have recently revealed a changing bacterial behaviour. For example, flagellar motility and chemotaxis-related genes were transcribed in the epiphytic phase, while genes controlling metabolism in the apoplast (X. Yu et al., 2013). In another study, gene expression of virulent and avirulent *P. syringae* strains was studied at different time points after inoculation of various *Arabidopsis thaliana* defence-related mutants. This work identified an iron response regulator that was induced at early infection stages, counteracting plant immunity (Nobori et al., 2018). Other time course transcriptomes in *P. syringae* have described an up-regulation of flagellar, chemotaxis and two-component system genes and a down-regulation of bacterial secretion systems and general metabolism at late infection stages in bacteria recovered from plants with pre-induced immunity compared to naïve plants (Lovelace et al., 2018). Together, these studies have started revealing the complex landscape of transcriptomic changes occurring over time during the course of a bacterial infection.

Due to the various environments it encounters along the infection process and because of its economic relevance, *R. solanacearum* is an excellent model to analyse gene expression in different plant tissues, which correspond to distinct phases of the infection process. Here, we have analysed the transcriptome of the cold adapted *R. solanacearum* UY031 at three different infection stages. We have used the economically important crop potato plant where the *R. solanacearum* UY031 was naturally identified a decade ago (Siri et al., 2011). Our data clearly shows that *R. solanacearum* genes behave dynamically inside the plant during the course of infection. We have identified stage-specific expression of virulence and metabolic genes, providing a new dynamic perspective of the *R. solanacearum* infection process.

Material and Methods

Bacterial strains and plant growth conditions

The highly aggressive *Ralstonia solanacearum* strain UY031 (phylotype IIB, sequevar 1) isolated from potato tubers in Uruguay (Siri et al., 2011) carrying the synthetic *luxCDABE* operon under the control of the *psbA* promoter was used in this study (Monteiro, Genin, et al., 2012). The luminescence allowed indirect but precise quantification of bacteria and to track bacterial

proliferation *in planta* (Cruz et al., 2014). Bacteria were routinely grown at 30ºC in rich B medium supplemented with 0.5% glucose (Monteiro, Genin, et al., 2012).

Solanum tuberosum cv. Desirée potato plants were propagated *in vitro* (Puigvert et al., 2017) and 2-week old apexes were transferred to a soil:silica sand mixture in a 1:1 ratio for RNA-seq sampling or moved to a substrate:perlite:vermiculite mixture in a 30:1:1 ratio for *in planta* visualisation. Plants were grown at 22ºC under long day (16 h / 8 h light/dark) conditions for 3 weeks.

Bacterial sampling

For liquid medium samples, bacterial cultures were set to an starting OD_{600} = 0.1 (10^8 CFU/ml) and grown for 5 h in rich B medium (10 g/L bacteriological peptone, 1 g/L yeast extract, 1 g/L casamino acids), until they reached exponential growth phase (OD_{600}~0.4-0.5). Bacteria were then centrifuged at 4ºC for 2 min at maximum speed and the pellet was immediately frozen in liquid nitrogen.

To assess bacterial colonisation levels, especially in asymptomatic plants, stems were placed under a luminometer to visualize bacterial densities within the vascular system, and only plants showing luminescence were used. To avoid bias of quorum sensing signals in the xylem stages and not in the apoplast, similar bacterial yields were infiltrated in potato leaves for the initial stage. Finally, to identify the best time point at which bacterial colonisation within xylem vessels of almost asymptomatic plants was most similar to that in dead plants, we monitored bacterial growth, luminescence and disease symptoms over time (Additional File 1A). As shown in Additional File 2A, bacterial densities recovered from the three *in planta* conditions are in the same order of magnitude (between 10^7 and 10^8 CFUs/ml). The *in vitro* reference condition corresponding to bacteria grown in liquid rich medium, was also obtained to better define *R. solanacearum* gene expression. We ensured that the difference of the final bacterial yields from the different conditions was not higher that one log (Additional File 2A). These conditions allowed us to obtain enough *R. solanacearum* RNA-seq reads to have a robust representation of the whole genome (Additional File 3). Principal component analysis revealed that these conditions are consistent among biological replicates and sensitive enough to detect biological differences between conditions (Additional File 2C).

To obtain more reproducible samples, leaf apoplast was used as a mimic condition of root apoplast, since it has been reported that *R. solanacearum* behaves similarly in these two apoplastic spaces (Hikichi, 2016).

To obtain leaf apoplast samples, bacterial cells from an overnight culture were washed with water and resuspended to a final concentration of $5x10^8$ CFU/ml. The aerial part of the plants

was vacuum-infiltrated for 30 sec to 1 min and the leaves were dried in paper towel before incubating the plants in the inoculation chamber (27ºC, 12 h / 12 h). After 6 hours, leaves were vacuum-infiltrated with sterile distilled water, dried in paper towel, rolled in a cut tip and centrifuged inside a 50 ml tube at 4ºC for 5 min at 2000 rpm. Apoplast fluid extract was pooled (each pool representing approximately 15 plants) and centrifuged at 4ºC at maximum speed for 2 min. Bacterial pellets were frozen in liquid nitrogen.

For early and late xylem samples, potato roots were injured with a 1 ml tip before inoculation. A total of 40 ml of a 10^8 CFU/ml *R. solanacearum* suspension was used to soil-inoculate each plant. After inoculation, plants were kept inside the inoculation chamber (27ºC, 12 h / 12 h) for 6 days (mean disease index = 0 - 1) for early xylem condition, or 10 days (disease index = 4 in all the plants) for late xylem condition. Plants were photographed in a Fuji Film LAS4000 light imager system to check individual infection levels and only plants showing luminescence were used. Stem pieces of 2 cm were cut from each plant, placed in a 1.5 ml tube containing 500 µl of sterile distilled water and centrifuged 2 min at maximum speed at 4ºC to release bacteria from the xylem vessels. In all cases, bacterial densities were measured by luminescence before freezing and dilutions were plated to measure CFUs before addition of 5% of an ice-cold transcriptional stop solution (5% [vol/vol] water saturated phenol in ethanol). This enabled normalisation of early or late xylem samples for bacterial concentrations comparable to those of apoplast and reference medium samples. Bacterial pellets were pooled together for each biological replicate and frozen in liquid nitrogen. Approximately 30 plants were used for each early xylem replicate and 7 plants for every late xylem replicate (Additional File 2A).

RNA extraction, sequencing and library preparation

Total RNA was extracted using the SV Total RNA Isolation System kit (Promega) following manufacturer's instructions for Gram-negative Bacteria. RNA concentration was measured with a ND-8000 Nanodrop and RNA integrity was validated for all samples using the Agilent 2100 Bioanalyzer. For rRNA depletion, 2.5 µg of total RNA were treated with the Ribo-zero (TM) magnetic kit for bacteria (Epicentre). Three biological replicates per condition were subjected to sequencing on a HiSeq2000 Illumina System apparatus using multiplexing and kits specially adapted to obtain 100 bp paired-end reads in stranded libraries. Rich media reference samples were sequenced by Macrogen Inc. In all other cases, RNA-sequencing was performed in the Shanghai PSC Genomics facility. Raw sequencing data will be available upon publication in the Sequence Read Archive under Bio Project: PRJNA660623 (accession codes SAMN15955133 to SAMN15955144).

Read alignment, mapping and differential gene expression analysis

RNA-seq raw data quality was evaluated using FASTQC (version 0.11.4, (Andrews et al., 2015)). *R. solanacearum* reads were mapped using Bowtie2 (version 2.3.3, (Langmead & Salzberg, 2013)) with stringent parameters (Puigvert et al., 2017) using as reference the completely sequenced genome of UY031 strain (Guarischi-Sousa et al., 2016). Alignment files were quantified with HTSeq-count (version 0.11.3, (Anders et al., 2015)) using NCBI's RefSeq sequences NZ_CP012687.1 (chromosome) and NZ_CP012688.1 (megaplasmid). The DESeq2 package (version 1.28.1, (Love et al., 2014)) in R ((R Team, 2017), ver. 3.6.3) was employed to perform differential expression (DE) analysis of high quality RNAseq reads. Genes with $|\log_2(\text{fold-change})| > 1.5$ and adjusted *p-value* < 0.01 were considered as DE *in planta* when compared to bacteria grown on liquid rich medium as reference condition (Additional File 2C). The UpSetR (Conway et al., 2017) R package was used to visualise the intersection of DE genes in the different *in planta* conditions.

Gene expression pattern clustering and enrichment analysis

To obtain expression profiles of *R. solanacearum* UY031 genes, a soft clustering analysis was performed using Mfuzz package (version 2.48, (Kumar & Futschik, 2007)) in R. Input data corresponds to the DE fold-change values yielded by DESeq2 of apoplast, early and late xylem samples normalised to the reference liquid rich medium. The cluster number was manually set at c=6. To be more stringent, a gene was considered to belong to a specific cluster if the gene was allocated in the same cluster in 30 out of 40 iterations with the membership value set to $\mu \geq 0.75$.

To further characterise the genes differentially expressed or belonging to any of the clusters, we looked for enriched Gene Ontology (GO) terms or Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways among our genes. Since no GO terms had been previously associated to UY031 strain genes, we used Blast2GO (Götz et al., 2008) software to annotate the UY031 genome. For the KEGG and GO enrichment analysis, we used the enricher function of the ClusterProfiler package (G. Yu et al., 2012) in R having previously created the TERM2GENE and TERM2NAME lists to do the hypergeometric test.

Because KEGG enrichment analysis is limited to a number of pre-established pathways or terms that do not include important virulence categories, and because pathogenicity-related terms in GO are too general and have not been widely used, we decided to create a manually curated category that we defined as "virulence and parasitic fitness (Additional File 4). This category included the T3SS and type III effectors, motility genes, exopolysaccharides secretion, phytohormone biosynthesis, ROS scavenging, nitrogen metabolism and cell-wall degrading enzymes. After defining the genes included in this category, we conducted a hypergeometric test using the R stats package on the differentially expressed genes or the gene clusters to find

out whether the "virulence and parasitic fitness" or any of the subcategories was overrepresented.

***In planta* visualisation of *R. solanacearum*.**

To visualize *R. solanacearum* bacterial cells in early (6 days post-inoculation, d.p.i) and late (10 d.p.i.) xylem stages, UY031 with the *psbA* constitutive promoter was fused to the GFP gene. This reporter strain was soil-inoculated with root wounding at OD_{600}=0.1 (10^8 CFUs/ml) in 3 week-old potato plants. Potato stem slices from the first node of infected plants with GFP-containing bacteria were observed in the SZX16 stereomicroscope equipped with a DP71 camera system (Olympus). Pictures were obtained using the following settings: GFP filter, 10 seconds exposure time, ISO 1/800. Control plants were soil-inoculated with water (Additional File 1B).

Results

***R. solanacearum* transcriptomes reflect four main genetic programmes inside the plant**

To elucidate the genes deployed by *R. solanacearum* throughout infection, we profiled the gene expression of strain UY031 in its natural susceptible potato host. We collected bacterial samples from the apoplast —a condition mimicking early root infection, when the bacterium traverses and multiplies in this compartment (Hikichi, 2016)— and from the xylem of infected plants at six and ten days post-inoculation, which correspond to the onset of the disease (early xylem) or to the final stages when plants are completely wilted (late xylem) (Additional File 2A and 1B). *R. solanacearum* plant infection through roots is highly variable due to stochastic changes in the physiological state of the plant, the initial inoculum and available root entry sites. To overcome this problem, we took advantage of a luminescent *R. solanacearum* reporter strain previously developed in our group to measure bacterial colonisation and we normalized values for tissues containing comparable bacterial loads at different times of infection (Cruz et al., 2014). The *in planta* transcriptomes were compared with that obtained from bacteria grown in liquid rich B medium, a reference condition known to repress many of the pathogen's virulence determinants (Arlat et al., 1992). Principal component analysis (PCA) of the transcripts from each sample showed a clear clustering of the biological replicates and a clear differentiation of the xylem samples from the reference and apoplast samples (PC1, explaining 65% of the variation) (Additional File 2C). Comparison of the *in planta* transcriptomes with that obtained in axenic growth in rich medium identified 418 differentially expressed genes (DEGs) in the apoplast, 531 in the early xylem and 922 in the late xylem ($\log_2$ fold change ≥ |1.5| and adjusted *p-value* ≤ 0.01). Of these genes, 226 and 192 were up- and

down-regulated, respectively, in the apoplast, 290 and 241 in the early xylem, and 378 and 544 in the late xylem (Figure 1A and Additional File 5).

Comparison of the DEGs in each *in planta* condition is in agreement with the previously published *R. solanacearum in planta* transcriptomic studies (Additional File 6). DE transcripts from the same UY031 strain retrieved from total RNAs of infected wild potato roots (Puigvert et al., 2017) showed up to 17-18% overlap with the apoplast condition and lower overlap with the other conditions assayed in the present study. This is logical, since the transcriptome previously obtained from roots of asymptomatic plants corresponds to an early time of infection where most bacteria grow apoplastically and only a small proportion of bacteria have already reached the xylem. The highest overlap (34% overlap in up- and 36% in down-regulated genes, respectively) was found between our early xylem conditions and the microarray transcriptome of the phylogenetically close strain UW551 isolated from tomato plants at a comparable infection time (onset of wilting symptoms) (Jacobs et al., 2012), which further validates our results (Additional File 6). The overlap is obviously lower with comparable transcriptomes obtained using the distantly related GMI1000 strain.

To discover the DEGs common or unique to the different plant environments, we analysed the shared genes among the different conditions studied. As can be observed in Figure 1A, two intersections (i.e. *in planta* and xylem) and two conditions (i.e. apoplast and late xylem) that correspond to bacterial growth in precise environments included most of the DEGs. On this basis, we defined four genetic programmes where *R. solanacearum* expresses exclusive gene sets: *in planta* (genes shared in all *in planta* conditions: apoplast, early and late xylem), the xylem (genes shared in early and late xylem), the apoplast, and the late xylem. Similarly, DE in all *in planta* conditions were 104 up- and 81 down-regulated genes. The differentially expressed genes in the xylem genetic programme (both time points analysed) included a total of 162 and 156 up- and down-regulated genes. Finally, 100 and 80 genes were, respectively, up- or down-regulated solely in the plant apoplast and 96 and 278 only in the late xylem condition, when plants are mostly dead. The remaining conditions or overlaps between conditions included fewer than 30 specifically DEGs (Figure 1A) and we did not consider them a proper “genetic programme”. Overall, as hinted by the PCA analysis, the apoplast showed the most divergent transcriptome of the *in planta* conditions, whereas the samples extracted from the xylem (early and late) were the most similar. However, a substantial fraction of genes was only differentially expressed in the late xylem (40% of those DE in this condition).

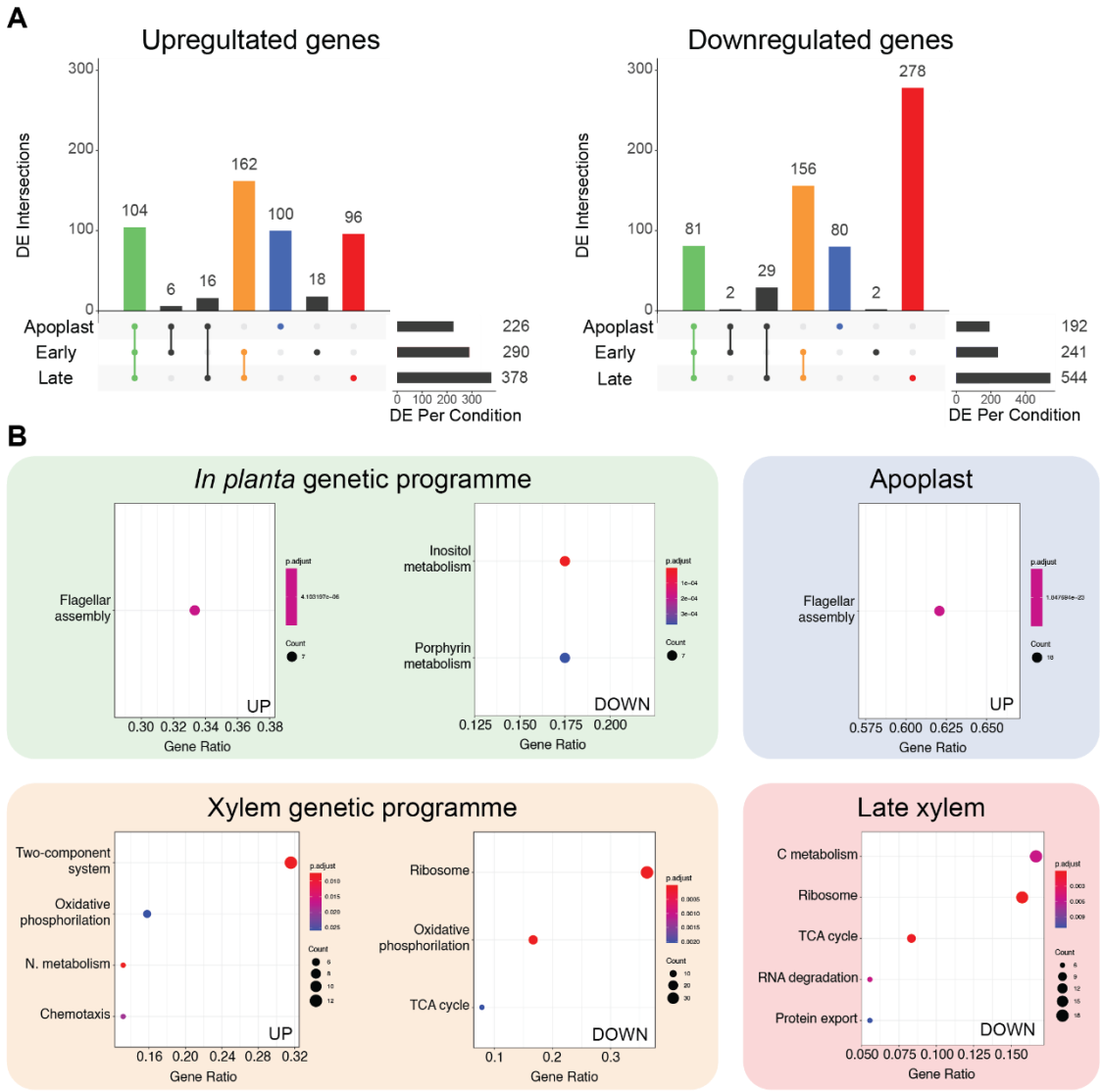


Figure 1. **Transcriptomic profile of *R. solanacearum* UY031 *in planta* environments**. **A**. Shared and unique DE genes across the three *in planta* conditions for the up-regulated (left) and down-regulated (right) genes. Each vertical bar plot represents the number of shared DE between the conditions indicated by the lines and dots in the schematic below. The horizontal bar plots on the right indicate the total DE genes per *in planta* condition compared to rich medium. **B.** For the intersection of Apoplast, Early and Late (*in planta* environment), Early and Late (Xylem environment), Apoplast and Late xylem alone, the list of genes was extracted and surveyed for enriched KEGG pathways. Dot plots of the enriched KEGG pathways for the up- (left) and down-regulated (right) genes in each environment is shown below. DE genes were identified with DEseq2 (*p-adj* > 0.01, log2FC±1.5) and plotted using the R package UpsetR.

R. solanacearum upregulates a variety of virulence factors in the plant genetic programme

Functional enrichment of gene annotations is a powerful tool to evaluate the genes involved in similar roles or pathways in each experimental condition. Thus, we investigated the enrichment of KEGG pathways and GO terms in the genes that appeared DE in all *in planta* conditions. Since the KEGG database contains metabolic pathways and terms specifically for prokaryotes, we focused on its categories for enrichment analysis. Among the genes up-

regulated in all *in planta* conditions, only the KEGG flagellar assembly pathway was enriched (Figure 1B). This result was confirmed by the GO enrichment analysis, where the bacterial flagellum-dependent cell motility term was similarly over-represented, together with transposase activity and DNA-mediated transposition (Additional File 7). On the other hand, the enriched KEGG terms amongst the genes down-regulated in all *in planta* conditions were all related to metabolism: inositol phosphate metabolism, and porphyrin and chlorophyll metabolism (Figure 1B), and the GO term cobalamin biosynthetic process (Additional File 7).

Manual curation of gene annotations enabled us to pinpoint a high number of pathogenicity-related functions up-regulated in all *in planta* conditions. These genes had been overlooked by the global enrichment analysis because virulence genes are not in a KEGG pathway and pathogenicity-related terms in GO are too general and have not been widely used. Thus, we used genomic and bibliographic information to create the gene category "virulence and parasitic fitness" for the UY031 strain and calculated its enrichment in all conditions or genetic programmes analysed in this work (see Methods). The new category included all genes encoding the type III secretion system (T3SS) and its associated effectors (T3Es), genes involved in motility, EPS and phytohormone biosynthesis, ROS scavenging, cell-wall degrading enzymes, and nitrogen metabolism (Additional File 4). As expected, the created "virulence and parasitic fitness" category was clearly enriched in the up-regulated genes in the *in planta* genetic programme (*p-value* = $1.4 \cdot 10^{-14}$). Detailed analysis of the subcategories included in "virulence and parasitic fitness" indicated that T3SS and T3Es (*p-value* = $2.4 \cdot 10^{-12}$) and motility (*p-value* = $5.7 \cdot 10^{-5}$) were also statistically enriched among the up-regulated genes. For instance, 20% (12 out of 60) of the genes annotated as T3Es were overexpressed in all *in planta* conditions. The enriched motility subcategory included a total of 11 genes, containing both flagellar and type IV pili. Similarly, the polygalacturonase gene *pglA,* encoding one out of the six cell-wall degrading enzymes in the genome was also up-regulated in the plant. Other virulence genes up-regulated in bacteria growing in any of the studied *in planta* conditions included *efe,* responsible for ethylene formation, the reactive oxygen species (ROS) scavenging superoxide dismutase *sodC*, and *epsR,* encoding the exopolysaccharide (EPS) repressor. Finally, only the EPS subcategory was under-represented *in planta* (*p-value* = $1.25 \cdot 10^{-2}$), which can be explained by the high expression of the exopolysaccharide synthesis operon in the reference rich medium (Steven J. Clough, Flavier, et al., 1997).

Flagellar genes and the upstream regulators of the T3SS are exclusively up-regulated in the apoplast

Once *R. solanacearum* has infected the roots of a susceptible host plant it must cross the root cortex through the apoplast. The KEGG flagellar assembly pathway was enriched in the genes exclusively up-regulated in the apoplast (Figure 1B). Similarly, the four GO terms referring to the flagellum (bacterial-type flagellum-dependent cell motility, bacterial-type flagellum basal body, bacterial-type flagellum and bacterial-type flagellum assembly) and phosphopantetheine binding were also enriched in this genetic programme (Additional File 7). A closer perusal of the list of up-regulated genes in the apoplast genetic programme also revealed that the "virulence and parasitic fitness" category was enriched (*p-value* = $4.2 \cdot 10^{-15}$). *PrhJ* and *hrpG*, key upstream regulators of the T3SS activation cascade (Brito et al., 1999), were overexpressed in this genetic programme. On the other hand, none of the downstream T3SS transcriptional activators and only two of 60 T3E genes (*ripE2* and *ripAD)* were exclusively up-regulated in this genetic programme. None of the KEGG pathways nor GO terms were enriched amongst the genes down-regulated in the apoplast.

***R. solanacearum* adapts to the xylem environment by inducing virulence, chemotaxis and nitrogen metabolism**

After travelling through the root apoplast, *R. solanacearum* crosses the Casparian strip, reaching the plant vasculature and heavily colonising the xylem vessels. As mentioned before, a substantial number of *R. solanacearum* genes was DE in the xylem genetic programme, both at early and late infection stages (Figure 1A). Almost one third (12 out of 38) of the genes with associated KEGG pathways differentially up-regulated in the xylem irrespective of the infection stage belonged to the enriched category two-component system (Figure 1B). This includes genes that participate in chemotaxis signal transduction, nitrate reduction, and oxidative phosphorylation. Three other categories were enriched in the genes up-regulated in the xylem: oxidative phosphorylation (six genes), bacterial chemotaxis (five genes) and nitrogen metabolism (five genes). The up-regulated nitrogen metabolism genes included nitrate transporters (*nark1*/2), enzymes involved in the denitrification pathway (*aniA*, *norB*) and in the dissimilatory nitrate reduction pathway (*narG/H/I, nirB/D*) as well as in reactive nitrogen species detoxification (*hmpX*). The enriched term bacterial chemotaxis included genes involved in different steps of swimming motility, including membrane chemosensors, signal transduction components (i.e. *cheZ1, cheA, cheR*) and flagellar motor genes (i.e. *motB*). The "virulence and parasitic fitness" category was also enriched in the xylem genetic programme

up-regulated genes (*p-value* = $8.8 \cdot 10^{-5}$). Amongst these genes were 9 out of 60 T3Es annotated in strain UY031 genome (*ripAE, ripY, ripAN, ripC1, ripN, ripAP, ripF2, ripBH,* and *ripS5),* and one out of six cell wall degrading enzymes (*pme)*. Other overexpressed genes in the category included 10 motility genes and the cytokinin biosynthesis gene *tzs*. Finally, amongst the 102 KEGG tagged down-regulated genes in the xylem, the enriched categories were: ribosome, oxidative phosphorylation and citrate (TCA) cycle (Figure 1B). GO enrichment in down-regulated genes similarly showed the over-represented categories translation, ribosome, structural constituent of ribosome, RNA binding, rRNA binding (Additional File 7). In summary, a large set of *R. solanacearum* genes was found DE in the xylem throughout infection, including up-regulation of nitrogen utilisation and virulence genes, such as T3Es and down-regulation of genes encoding the citrate cycle enzymes and the electron transport chain.

Temporal expression profiles reinforce the existence of specific genetic programmes in the apoplast and the xylem.

The findings described so far strongly suggest that *R. solanacearum* expresses specific sets of genes at each step of the infection process. To better understand this dynamic process, we obtained the expression profiles of the *R. solanacearum* UY031 genes in the three *in planta* conditions: apoplast, early and late xylem. To this end, fold-change values of DE genes in each condition in relation to growth in rich culture medium were used as input to the Mfuzz clustering package. Six different gene expression profile clusters were identified according to the condition or temporal progression, considering that the apoplast is the earliest stage during infection, followed by early and late xylem (Figure 2). According to this, the profile named “specific apoplast” contained 807 genes up-regulated in the apoplast but down-regulated in early and late xylem (Figure 2A), and the profile “specific xylem” contained 1286 genes down-regulated in the apoplast but up-regulated in the other conditions (Figure 2B). We identified two additional profiles, including genes that continuously decreased (561 genes up-regulated in the apoplast with transcripts gradually decreasing in xylem over time) (Figure 2C) or increased (334 genes, opposite profile) (Figure 2D) their expression over the infection period. Finally, the genes specifically repressed (Figure 2E) or induced (Figure 2F) in the early xylem that showed the opposite trend in the apoplast and late xylem were 105 and 107, respectively.

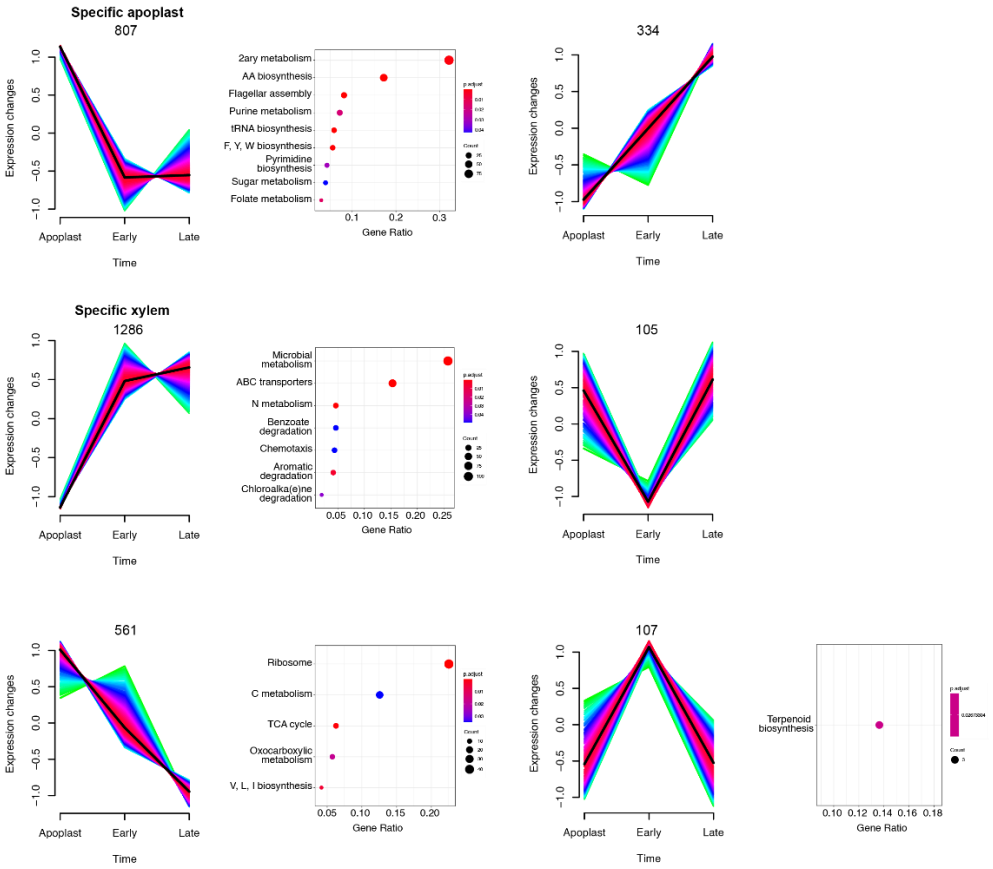


Figure 2. **Gene expression dynamics of *R. solanacearum* throughout infection**. Six clusters were obtained through Mfuzz clustering of log2-fold-change data of the apoplast, early and late xylem conditions normalised to the reference rich liquid media. Clusters include the genes (number indicated above each graph) with a membership higher than 70% and consistently associated to the same cluster on at least 30 out of 40 iterations. The list of genes associated to each cluster was extracted and surveyed for enriched KEGG pathways. Dot plots of the enriched KEGG pathways in each cluster is shown next to the cluster.

To unveil the biological functions behind each expression profile, we performed enrichment analyses. Enriched KEGG pathways in the "specific apoplast" expression profile included various biosynthetic processes, especially biosynthesis of secondary metabolites (99 out of 308 tagged genes) and related pathways such as biosynthesis of amino acids (53 genes) and flagellar assembly (25 genes) (Figure 2A). Our manually-defined motility subcategory was enriched in this expression profile (*p-value* = $1.78 \cdot 10^{-2}$). In the "specific xylem" profile, the KEGG enrichment analysis yielded terms related with metabolism adaptation such as microbial metabolism in diverse environments (106 out of 411 tagged genes), ABC transporters (63 genes), and nitrogen metabolism (19 genes) among others (Figure 2B). Our manually-defined subcategories T3SS & T3Es (*p-value* = $5.2 \cdot 10^{-3}$), phytohormones (*p-value* = $2.5 \cdot 10^{-3}$) and nitrogen metabolism (*p-value* = $2 \cdot 10^{-6}$) were also significantly enriched in this profile. KEGG enriched terms within the continuous decrease profile were linked to transcription and carbohydrate metabolism such as ribosome (43 out of 191 tagged genes) and carbon

metabolism (24 genes) (Figure 2C). Finally, the profile containing genes with specific up-regulation in the early xylem, was enriched in the ubiquinone and other terpenoid-quinone biosynthesis pathway (3 out of 22 tagged genes). The subcategory T3SS & T3Es was significantly enriched in this expression profile as well (*p-value* = $1.34 \cdot 10^{-2}$), containing genes such as the master regulator *hrpB,* and three T3 effectors (Figure 2F). GO enrichment analysis confirmed these results, showing over-represented categories with similar biological functions (Additional File 8).

R. solanacearum specifically activates different sets of virulence factors in different plant environments

As described above, key virulence activities were induced in specific plant environments or at specific disease stages. To analyse in further detail the genes in this "virulence and parasitic fitness" (Additional File 9) and its subcategories we graphically represented their normalised read counts in all assayed conditions, including the reference condition in rich medium. This provided an unbiased view on the gene expression data avoiding the effect of the reference condition in the DESeq analysis. Detailed observation of gene expression values in heatmap representations for the T3SS (*hrp* and *hrc* genes) and T3E (*rip* genes) reinforced the above-described enrichment in various genetic programmes or conditions (Figure 3). Both the *rip* T3Es and the *hrp/hrc* genes displayed a very homogeneous expression pattern with high expression levels in the xylem genetic programme (early and late) and low expression levels in the apoplast. The only exceptions among the effectors were the two *ripI* genes, with low expression levels in all studied conditions, *ripE2*, with higher expression in the apoplast, and a cluster of effector genes (i.e. *ripAD* and *ripD*), showing high transcript levels in all conditions (Figure 3).

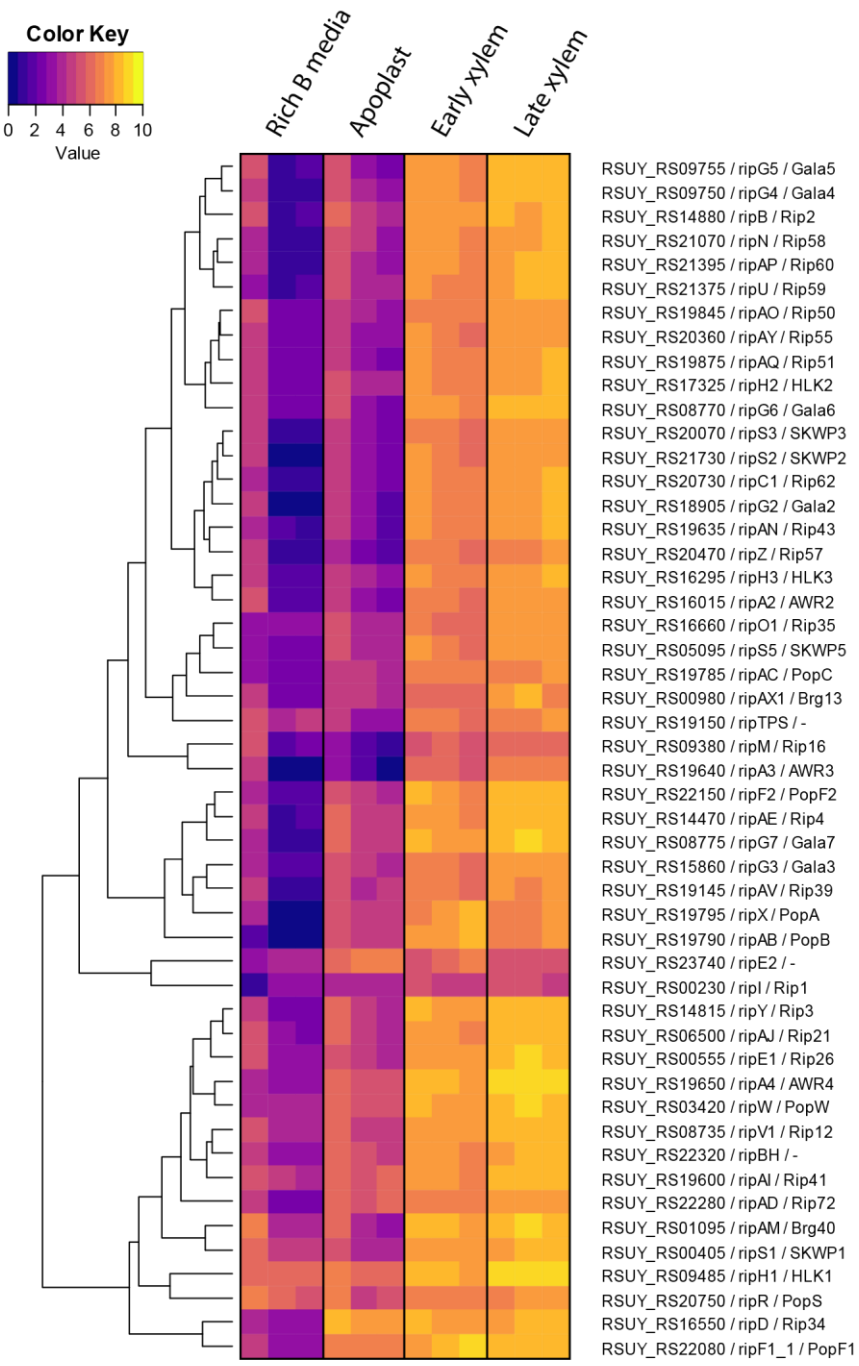


Figure 3. **T3E gene expression profile**. Heatmaps showing the normalised transcripts per million (TPM) of the genes coding the 60 T3E described in *R. solanacearum* UY031 in the reference condition and *in planta* apoplast, early and late condition. Only putatively functional T3E genes are included according to Peeters *et al*., 2013.

Heatmap visualisation of the normalised transcriptomic data also indicated that flagellar genes —essential for swimming motility— were highly expressed in all *in planta* conditions, but to a higher extent in the apoplast (Figure 4 top panel). This is in accordance with the enrichment of this category *in planta* and in the late xylem genetic programmes up-regulated genes, as well as in the specific apoplast profile. The *pil* twitching motility genes encoding type IV pili

followed a similar trend, although their expression was more similar in the apoplast and the xylem (Figure 4 bottom panel), suggesting that the bacterium is using the pilus appendix in all assayed plant environments. Exceptions to this trend were the flagellar genes (i.e. *fliM, fliS, fliD, fliT, motA, motB, fliC, fliO*) and the type IV pilus genes (e.g. *pilE1, pilY1, pilW, pilV, pilX*), which were down-regulated in the apoplast compared to the xylem genetic programme. The genes encoding chemotactic sensors and chemotaxis signal transduction proteins showed low expression levels in the apoplast and progressive induction in the early and late xylem conditions, (Additional File 10), in accordance with the enrichment of these specific genes in the late xylem genetic programme.

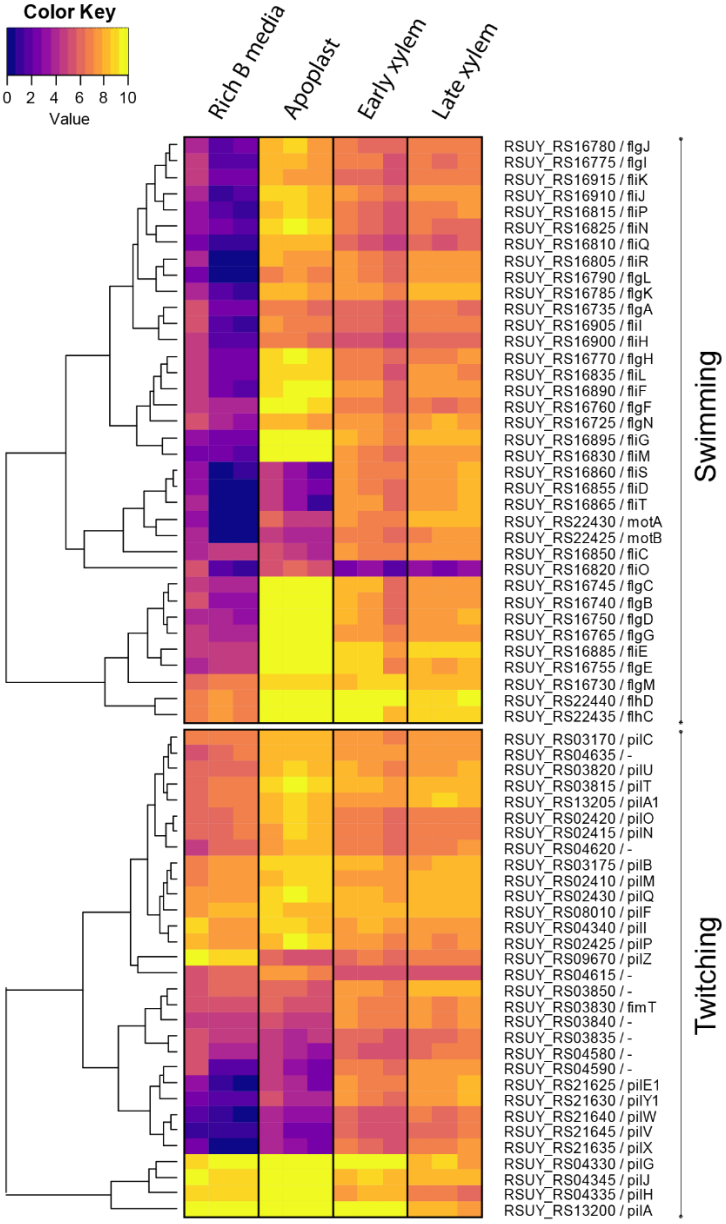


Figure 4. **Motility gene expression profile**. Heatmaps showing the normalised transcripts per million (TPM) of the genes involved in motility in the reference condition and *in planta* apoplast, early and late condition. Two heatmaps are shown to differentiate the swimming (top panel) and twitching (bottom panel).

Finally, all the UY031 genes that synthetize the plant hormones ethylene (*efe*), cytokinin (*tzs*), and auxin (*RSUY_RS1835* to *RSUY_RS18970*) (Valls et al., 2006) were highly expressed in the xylem genetic programme and to a lower extent in the apoplast, *efe* and *tsz* displaying a more sustained expression in the apoplast. This stable expression in all *in planta* conditions was also observed in the differential expression analysis (Additional File 11).

Discussion

R. solanacearum gene expression displays a behavioural differentiation into four plant genetic programmes that develop over time during in planta infection.

Previous *R. solanacearum* transcriptomic studies compared gene expression profiles obtained using a specific *in planta* condition, such as root apoplast (Puigvert et al., 2017) or early xylem colonisation (Jacobs et al., 2012), to reference bacteria grown in rich medium. In our study, we analysed the whole infection process, including three different *in planta* conditions: apoplast, early and late xylem, which typify paradigmatic stages of infection. Intersection of the DEGs of each of the three *in planta* experimental conditions showed that most of the DEGs of *R. solanacearum* during the infection are grouped in four biologically relevant genetic programmes: genes commonly DE in all *in planta* conditions, genes exclusively DE in the apoplast, genes expressed in the xylem at any stage of the disease and genes exclusively DE in the xylem when plants are already wilted (Figure 1). One of the previous transcriptomic studies sampled bacteria from plants 5 days post-inoculation (Jacobs et al., 2012), similar to our early xylem condition. With the addition of our novel late xylem condition 10 days after inoculation, and the apoplast condition, we provide a more detailed expression landscape of *R. solanacearum*, encompassing important different stages of the infection process. To study the transcriptomic data from a temporal perspective, we clustered the DEGs based on their expression profile across the three *in planta* experimental conditions (i.e. apoplast, early and late xylem) (Figure 2). Reinforcing the concept of the specific behaviour of *R. solanacearum* according to different genetic programmes, the largest number of DEGs appeared exclusively up-regulated in the xylem genetic programme (1286 genes) and the apoplast (807 genes). This finding confirms that *R. solanacearum* has different sets of genes that are successively deployed to infect the plant and adapt to the environments encountered along the infection. Here, we carefully investigated the expression pattern of the most important virulence factors to elucidate the bacterial strategies used to rewire the plant environments to its own benefit.

T3Es expression is prevalent throughout the *in planta* infection process, especially in the xylem.

The T3SS is the main pathogenicity determinant in *R. solanacearum*, as *hrp* mutants are completely avirulent (Boucher et al., 1985). The T3SS is tightly regulated by a transcriptional regulatory cascade that contains the constitutive receptor and transducer elements PrhA and PrhR and the transcriptional regulators PrhJ, HrpG and HrpB (Brito et al., 1999). Interestingly, in this work we found that this cascade appears sequentially induced during infection. As depicted in Figure 5, *prhI* and *prhJ* are exclusively induced in the apoplast, *hrpG* expression also peaks in this environment but is sustained at lower levels in the early xylem and *hrpB* is expressed in the apoplast but highly induced in the early xylem, preceding the expression of the T3SS and most T3E, which is maximal at all xylem stages (Additional File 12).

Our gene expression dataset also shows that most of the 60 T3Es are highly induced in the xylem genetic programme, confirming our previous results (Monteiro, Genin, et al., 2012) that challenged the view of T3Es as key only early after infection (Genin et al., 2005; Yoshimochi et al., 2009). In agreement our finding that almost all T3Es are simultaneously expressed in the xylem, a recently published study showed that deletion of 42 *R. solanacearum* T3E genes was required to compromise virulence of the bacteria on tobacco and eggplant and proliferation inside the xylem (Lei et al., 2020).

.

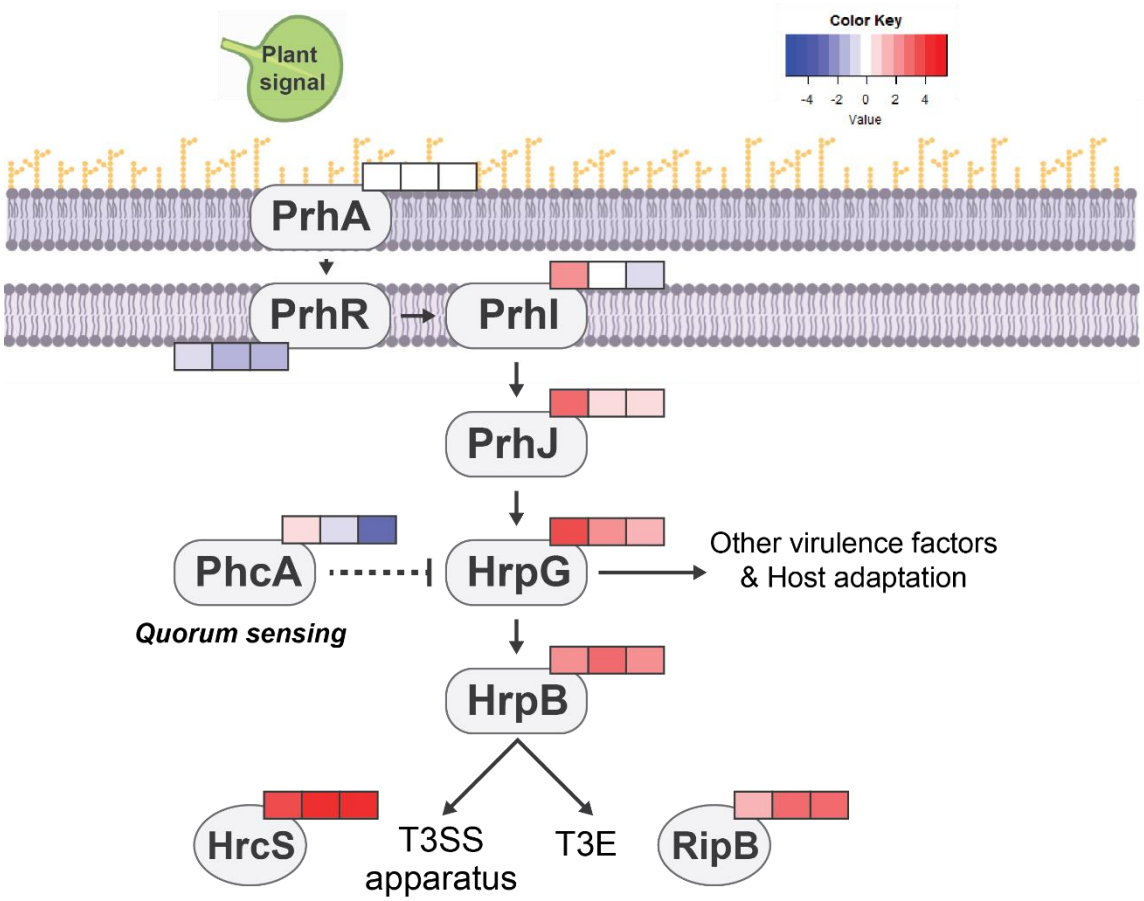


Figure 5. **T3SS regulatory cascade**. Log2 fold-change expression profile in the Apoplast, Early and Late Xylem of the genes involved in the T3SS regulatory cascade and downstream activated genes. The log2fold-change is indicated by the colour gradient shown in the Colour Key.

Interestingly, all *R. solanacearum* T3Es belonging to gene families (*PopA/B/C*, *AWR2/3/4/5_1/5_2*, *SKWP1/2/3/5/7*, *HLK1/2/3* and *GALA2/3/4/5/6/7*) (Poueymiro & Genin, 2009) were clearly induced in the xylem throughout infection (Figure 3). The *GALA* effectors (e.g. *ripG2 to ripG7*) and the *AWR* effectors (e.g. *ripA2 to RipA5*) have both been shown to be collectively required for full bacterial virulence and to target the proteasome or inhibit the Target Of Rapamycin (TOR) pathway, respectively (Popa et al., 2016; Remigi et al., 2011; Solé et al., 2012). Their expression pattern suggests that these biochemical activities are likely carried out in the xylem. Similarly, The T3E *ripAY*, which has been proven to impair the redox status of the plant cell degrading glutathione through its gamma-glutamyl cyclotransferase activity (Fujiwara et al., 2016; Mukaihara et al., 2016; Sang et al., 2018; Wei et al., 2017), was clearly induced in the xylem (Figure 3). This points to the xylem as a stressing redox environment *R. solanacearum* must cope with.

In contrast, a few T3Es showed alternative induction patterns to the one described above. For instance, *ripE2* can be clearly classified as an "early effector" since it was highly induced in the apoplast compared to the other infection stages, while *ripD* and *ripAD* were highly induced in all *in planta* infection stages (Figure 3). *RipD* localizes in vesicle-like structures and blocks the flg22-induced ROS response in *Nicotiana benthamiana* (Jeon et al., 2020). This fact, linked with its high expression in the apoplast and the activation of flagellar genes in this condition, suggests that *R. solanacearum* counteracts flg22 plant defence responses from the first stages of infection onwards. On the contrary, *ripI,* which was shown to enhance plant production of gamma-aminobutyric acid (GABA), was lowly expressed in all *in planta* conditions (Figure 3) (Xian et al., 2020). Although GABA catabolization by *R. solanacearum* enhances its infection capacity, the overproduction of GABA in plant cells in the absence of sufficient bacteria to consume it has been shown to induce cell death (Xian et al., 2020). Therefore, we hypothesize that RipI expression inside the plant must be tightly regulated to induce the production of nutrients without triggering plant stress signals.

***R. solanacearum* modulates twitching and swimming in different plant environments.**

R. solanacearum uses two types of motility during the colonisation of plant tissues: swimming (Tans-Kersten et al., 2004) and twitching (Kang et al., 2002b). Swimming motility is an individual bacterial movement through liquid environments in which flagella rotate by an ATPase-driven motor that is directed by chemosensor proteins (Sampedro et al., 2015). Previous research showed that both flagella (*fliC*) and chemosensor (*cheA* and *cheW*) mutants were less virulent than the wild-type *R. solanacearum*, demonstrating that not just the flagellar movement but also the ability to direct it are essential for full virulence *in planta* (Yao & Allen, 2006). Interestingly, full virulence was restored when the chemotactic mutants were directly inoculated in the plant stem, indicating that swimming motility is of crucial importance at the very early stages of infection (Yao & Allen, 2006). In our data (Figure 4), most of the flagellar-encoding genes were highly induced in the apoplast and, to a lower extent, later in infection (early and late xylem), supporting the previously mentioned hypothesis. A small subset of flagellar genes including the motor (*motA, motB*), the flagellin subunit (*fliC*) and the filament cap (*fliD*) among others showed low expression in the apoplast, for which we have no plausible explanation.

R. solanacearum displays twitching motility, which involves the extension and retraction of type IV pili to move on solid or viscous surfaces (Mattick, 2002). This motility is involved in natural transformation, biofilm formation and virulence (Kang et al., 2002b). Inactivation of the

genes encoding the pilin protein (*pilA*), the secretin involved in the pilus extrusion (*pilQ*) or the protein required for pilus retraction (*pilT*) reduced *R. solanacearum* virulence (H. Liu et al., 2001). In our transcriptomic data, twitching motility genes showed a similar expression pattern than swimming motility, but they were less induced in the apoplast and their expression was often maintained in early and late xylem (Figure 4). This emphasizes the importance of twitching motility throughout the plant infection process, as showed by the effect on virulence of *pil* deletion mutants (Corral et al., 2020; Kang et al., 2002b; H. Liu et al., 2001). Finally, *pilI*, which encodes the type IV pili chemosensor protein, was especially induced in the apoplast (Figure 4 bottom panel), in agreement with our recent findings that it is involved in virulence especially during the early infection stages (Corral et al., 2020).

***R. solanacearum* specifically activates different nitrogen metabolism genes to thrive in the xylem.**

R. solanacearum encounters a hypoxic environment in the plant xylem, which could limit its growth as the bacterium usually uses oxygen as the main terminal electron acceptor. However, the xylem contains an optimal concentration of nitrate that *R. solanacearum* can use as terminal electron acceptor to maintain its growth rates in this environment (Dalsing et al., 2015). Our gene expression dataset shows a faint induction of the nitrogen metabolism in the apoplast, reaching its expression peak in the xylem (Figure 6). When nitrate is available in the extracellular space, it diffuses the outer membrane and is imported to the cytoplasm by NarK1/2. Once nitrate enters the cytoplasm, the nitrate reductase (NarG/H/I) converts it to nitrite and then to ammonia through nitrite reductase (NirB/D). We found both the transporters- and reductase-encoding genes induced in the xylem (Figure 6), suggesting that both import and dissimilatory nitrate reduction are active in this compartment (Figure 6).

Nitrite diffusing back to the periplasm allows *R. solanacearum* to perform denitrification, first by reducing nitrite to nitric oxide via the nitrite reductase AniA and finally by reducing nitric oxide to nitrous oxide via the nitric oxide reductase NorB. Expression of these denitrification pathway genes is also induced in the xylem (Figure 6), suggesting that *R. solanacearum* has de ability to detoxify the reactive nitrogen species produced during nitrate dissimilatory pathway in the anaerobic xylem vessels (Dalsing et al., 2015).

Moreover, *R. solanacearum* can also incorporate nitrogen to its central metabolism through the assimilatory nitrate reduction. The nitrate present in the cytoplasm is reduced to nitrite by NasA/B. A previous study showed that nitrate assimilation was essential for initial root attachment but was dispensable for growth, virulence, and competitive fitness (Dalsing &

Allen, 2014). The fact that *nasA* is induced in the xylem and not in the apoplast is in disagreement with these results and may indicate strain- or condition-specific roles of N genes in *R. solanacearum*. Finally, the nitric oxide anion in the cytoplasm can be detoxified using HmpX, whose expression is also highly induced in the xylem genetic programme (Figure 6), an indicator of a highly active N metabolism in this plant environment.

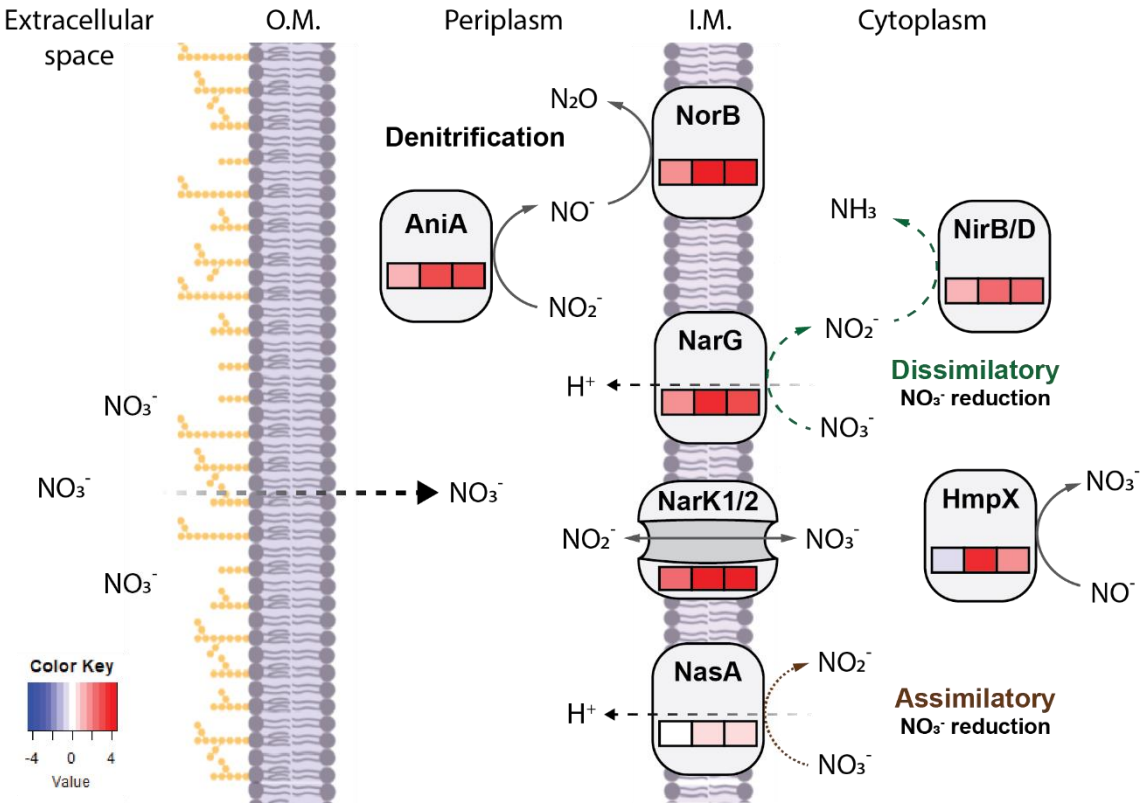


Figure 6. **Nitrogen metabolism expression profile**. Log2 fold-change expression profile in the Apoplast, Early and Late Xylem of the genes involved in the denitrification (*aniA, norB*), assimilatory (*nasA*), dissimilatory (*narK1/2, narG, nirB/D*) and nitrite detoxification (*hmpX*) pathways of nitrogen in *R. solanacearum* UY031. The log2fold-change is indicated by the colour gradient shown in the Colour Key. O.M = Outer membrane, I.M. = Inner membrane.

Phytohormone and ROS scavenging enzymes are expressed along the infection

R. solanacearum genome codes for phytohormone biosynthetic genes that drive the production of auxin (Valls et al., 2006), cytokinin (Genin & Denny, 2012) and ethylene (Freebairn & Buddenhagen, 1964). Interestingly, bacterially-produced auxin was described to block plant defences by the plant pathogen *Pseudomonas syringae* pv. *savastanoi* (Robinette & Matthysse, 1990) and ethylene was involved in wilting development in the pathosystem *A.*

thaliana-R. solanacearum (Hirsch et al., 2002). In this study, we observed induction of the cytokinin (*tzs*) and the ethylene (*efe*) biosynthetic genes as well as the auxin operon in the xylem (Additional File 11). Apoplastic induction of the master regulator *hrpG*, which also controls auxin and ethylene synthesis genes (Valls et al., 2006), precedes the xylematic expression of phytohormone biosynthesis genes as was observed for the T3SS (Figure 5).

After pathogen infection, plant cells respond with ROS production to create a hostile environment against the bacterium (Bolwell, 2002). Interestingly, *R. solanacearum* contains several genes that code for ROS scavenging enzymes, helping the bacterium survive in the plant apoplast and xylem (Flores-Cruz & Allen, 2009). Amongst them, alkyl hydroperoxide reductase genes (*ahpC1/C2/D/F*) were mostly induced in the xylem (Additional File 13). Several studies have linked the induction of *ahp* genes in biofilm-forming cells in different bacterial pathogens, contributing to protection against oxidative stress, epiphytic survival and attachment in the intercellular spaces or to the xylem vessels (Büttner & Bonas, 2010b; Jang et al., 2016; Oh & Jeon, 2014; Panmanee & Hassett, 2009; Wasim et al., 2009).

Conclusions

In summary, we performed a transcriptomic analysis of *R. solanacearum* at different infection stages in potato plants. DEG analysis revealed that *R. solanacearum* deploys specific gene expression programs in four different genetic programmes (i.e. *in planta*, apoplast, xylem, late xylem). Functional enrichment analysis showed that *R. solanacearum* has the highest expression of motility genes in the apoplast, while the majority of T3Es and nitrogen metabolism genes are highly induced in the xylem environment. This study provides for the first time a dynamic gene expression landscape of the bacterial plant pathogen *R. solanacearum* and is a first step towards the transcriptomic characterisation of its complete infection cycle.

Abbreviations

CFU: colony forming units

Cv: cultivar

d.p.i.: days post inoculation

DEG: Differentially Expressed Genes

DI: Disease index

DNA: desoxyribonucleic acid

EPS: exopolysaccharide

GABA: gamma-aminobutyric acid

GFP: Green Fluorescent Protein

GO: Gene Ontology

Hrp: hypersensitive response and pathogenicity

IVET: *in vivo* expression technology

KEGG: Kyoto Encyclopedia of Genes and Genomes

OD: Optical Density

PCA: principal component analysis

RLU: Relative Light Unit

RNA: ribonucleic acid

ROS: Reactive Oxygen Species

rRNA: ribosomal ribonucleic acid

T3E: Type 3 Effector

T3SS: Type III Secretion System

TCA: tricarboxylic acid

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files and the supporting datasets are available in Sequence Read Archive repository under Bio Project: PRJNA660623 (accession codes SAMN15955133 to SAMN15955144) in https://www.ncbi.nlm.nih.gov/sra.

Competing interests

The authors declare that they have no competing interests

Funding

This work was funded by project PID2019-108595RB-I00 from the Spanish Ministry of Economy and Competitiveness. We also acknowledge financial support from the "Severo Ochoa Program for Centres of Excellence in R&D" 2016-2019 (SEV-2015-0533) and the CERCA Program of the Catalan Government (Generalitat de Catalunya). R. de Pedro-Jové received FI and FPU fellowships from Generalitat de Catalunya and the Spanish Ministry of Economy and Competitiveness. P. Sebastià received the support of a fellowship (code is LCF/BQ/IN17/11620004) from la Caixa Foundation (identifier [ID] 100010434). This project has received funding from the European Union's Horizon 2020 research and innovation program under Marie Skłodowska-Curie grant agreement no. 713673. M. Puigvert received an APIF doctoral fellowship from Universitat de Barcelona and travel fellowship funded by Fundació Montcelimar and Universitat de Barcelona to carry out a short stay in J.C. Setubal's lab. J.C. Setubal holds a CNPq senior researcher fellowship. J.S. Monteiro is funded by a FAPESP doctoral fellowship under process number 2019/05287-2. A.P. Macho is funded by the Chinese Academy of Sciences and the Chinese 1000 Talents Program. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

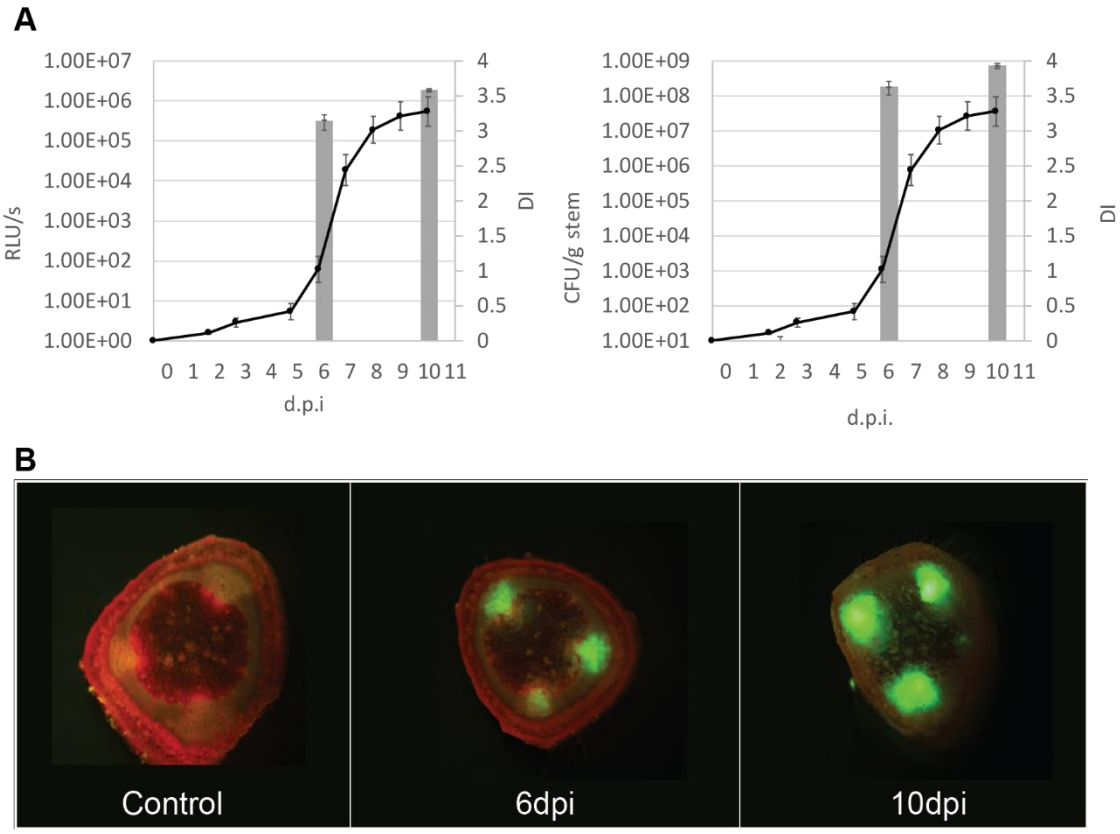
Authors' contributions

MV and JCS designed the study. APM sequenced the bacterial samples. RdeP, PS and MP performed the experimental work. RdeP, PS, MP, NSC, JSM, JCS, APM and MV analysed the data. RdeP, PS, MP, and MV wrote the paper. All authors have read and approved the manuscript.

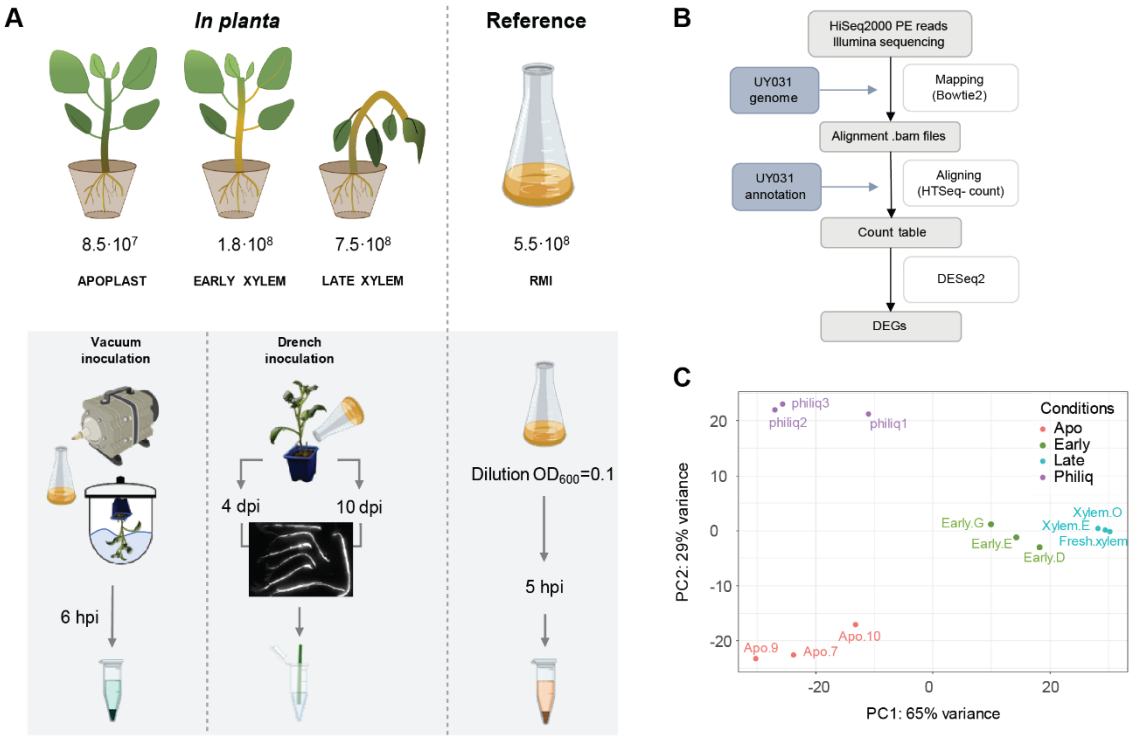
Acknowledgements

We thank C. Balsalobre and C. Madrid from the University of Barcelona and L. Tondo and E. Orellano from CONACYT Rosario for their useful advice, and CM Piroupo for help in submitting the sequence datasets to the Short Read Archive.

SUPPLEMENTARY MATERIAL



Additional File 1. ***R. solanacearum*** **reporter strains and bacterial growth show equivalent infection rates** A) Luminescence levels or bacterial growth and symptom development in potato plants were monitored over time to detect the precise time points at which similar bacterial yields but different symptoms could be detected. The disease index scale (DI) ranges from 0 to 4 being 0 symptomless plants and 4 plants completely wilted. (B) GFP-labelled bacteria were monitored at the sampled time points in potato plants. RLU = Relative light units.



Additional File 2. **RNAseq experimental set-up and bioinformatic pipeline.** (A) Experimental set-up for the three *in planta* conditions, corresponding to an early (leaf apoplast), mid (xylem from asymptomatic plants) and late stages (xylem from dead plants) of the disease. As reference condition, bacteria grown in rich liquid media were used. The average of bacterial yields recovered in each condition are indicated as CFU/ml. The grey background section of the figure contains the representation of how bacteria was enriched in each condition (see M&Ms). (B) Transcriptomic analysis pipeline. (C) Two-dimensional Principal Component Analysis representation of the expression data of the conditions' biological replicates used in the study.

Sample ID	Total reads	Aligned reads	% Aligned reads
Apoplast1	21879209	18225945	83.30
Apoplast2	26766576	22641780	84.59
Apoplast3	24945027	20106785	80.60
Early xylem1	23554394	19887599	84.43
Early xylem2	24102624	20115780	83.46
Early xylem3	25157995	18506738	73.56
Late xylem1	23270138	21909969	94.15
Late xylem2	23309741	21562241	92.50
Late xylem3	24741904	23166640	93.63
RMliq1	28623378	20467036	71.50
RMliq2	32546088	28018378	86.09
RMliq3	30741621	26841635	87.31

Additional File 3. **Proportion of reads aligned to *R. solanacearum* UY031 genome.** Total number of reads obtained in each biological replicate for each condition (first column). Total number of reads aligned to *R. solanacearum* UY031 genome (second column). Proportion of reads aligned to *R. solanacearum* genome expressed as percentage (third column).

https://drive.google.com/drive/folders/1E9W0H4Js0-CijGDVsQgTT3kxQF0kjaFV?usp=sharing

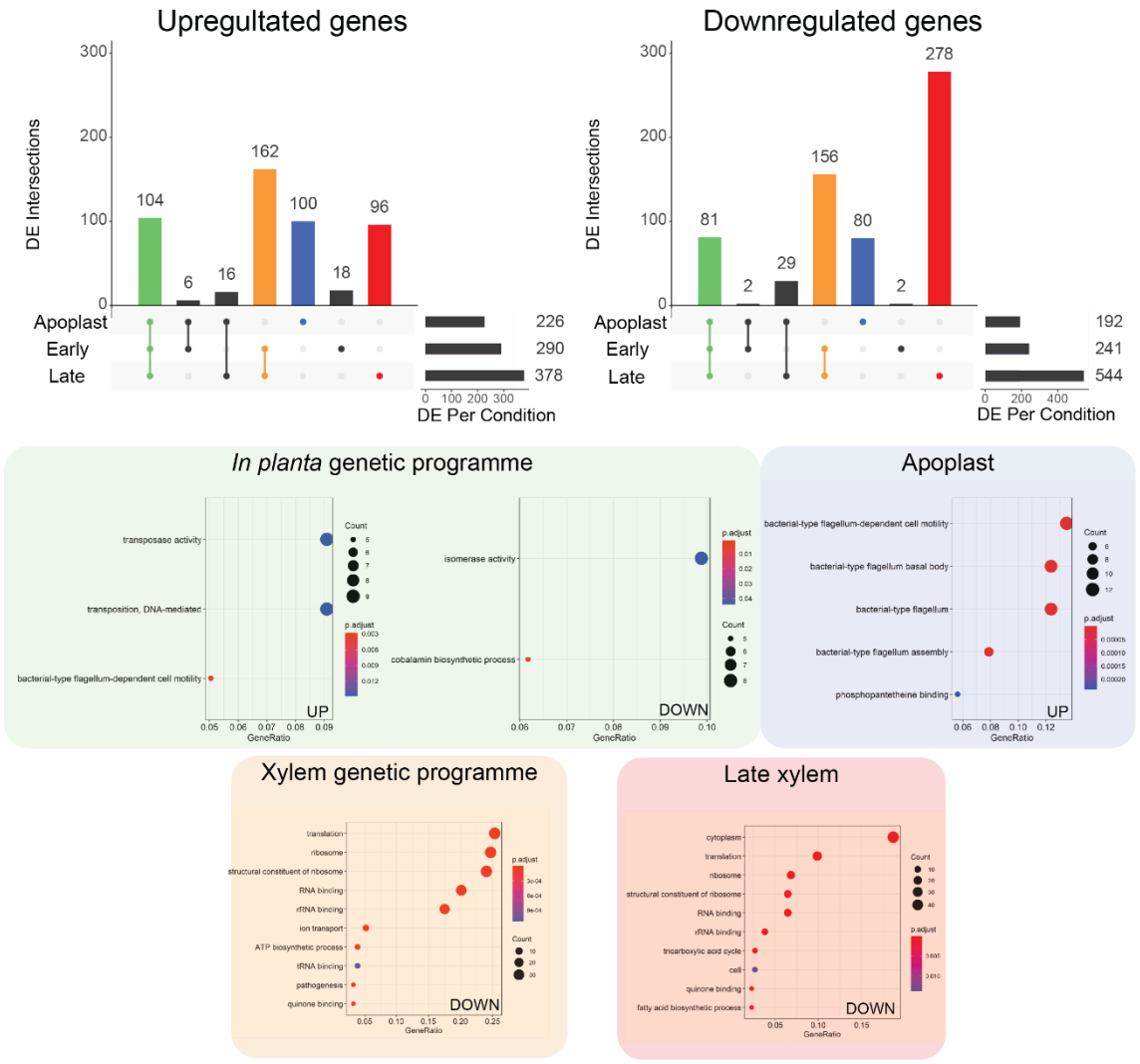
Additional File 4. "**Virulence and parasitic fitness" manually defined category.** Genes belonging to specific virulence categories (T3SS & T3Es, Motility, ROS scavenging enzymes, phytohormone biosynthesis, EPS, nitrogen metabolism, cell wall degrading enzymes) of *R. solanacearum* are listed showing information related to: UY031 NCBI locus tag (first column), gene name (second column), gene description (third column), category, (forth column), reference (fifth column). See **Note** (end of Chapter 1)

https://drive.google.com/drive/folders/1E9W0H4Js0-CijGDVsQgTT3kxQF0kjaFV?usp=sharing

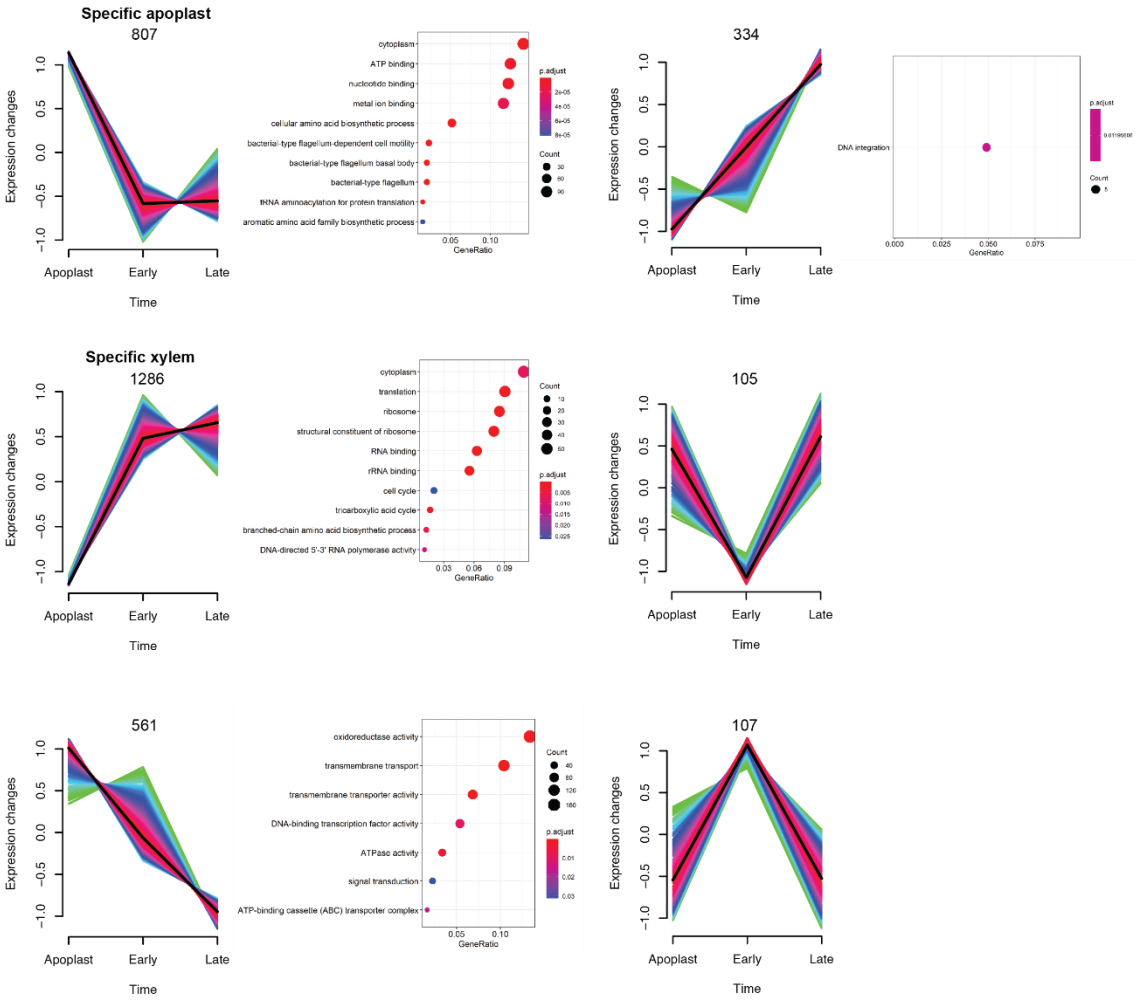
Additional File 5. **DEGs in the three *in planta* conditions.** Differentially expressed genes of *R. solanacearum* in apoplast, early and late xylem compared to liquid rich medium obtained with DESeq2 (*p-adj* > 0.01, log2FC±1.5). See **Note** (end of Chapter 1)

Induced - UP			Repressed - DOWN				
Apoplast	Early Xylem	Late Xylem	Apoplast	Early Xylem	Late Xylem		Ref
18% (40/226)	**9%** (27/290)	**5%** (20/378)	**0%** (0)	**1%** (3/241)	**5%** (26/544)	**RNAseq UY031 - Potato root UP**	(36)
0% (0)	**0%** (1/290)	**0%** (1/378)	**17%** (32/192)	**9%** (21/241)	**5%** (26/544)	**RNAseq UY031 - Potato root DOWN**	(36)
33% (74/226)	**34%** (100/290)	**27%** (102/378)	**6%** (11/192)	**3%** (8/241)	**2%** (12/544)	**µarray UW551 - Tomato UP**	(14)
1% (2/226)	**1%** (4/290)	**1%** (4/378)	**32%** (61/192)	**36%** (86/241)	**26%** (142/544)	**µarray UW551 - Tomato DOWN**	(14)
13% (30/226)	**20%** (58/290)	**15%** (56/378)	**5%** (9/192)	**3%** (8/241)	**2%** (9/544)	**µarray GMI1000 - Tomato UP**	(14)
1% (2/226)	**1%** (2/290)	**1%** (2/378)	**16%** (31/226)	**21%** (50/241)	**16%** (86/544)	**µarray GMI1000 - Tomato DOWN**	(14)
8% (17/226)	**9%** (27/290)	**7%** (27/378)	**6%** (12/226)	**5%** (11/241)	**3%** (16/544)	**RNAseq GMI1000 - Tomato UP**	(42)
5% (12/226)	**5%** (14/290)	**4%** (15/378)	**15%** (29/226)	**8%** (19/241)	**12%** (63/544)	**RNAseq GMI1000 - Tomato DOWN**	(42)

Additional File 6. **Overlap of DEGs in apoplast, early and late xylem compared to previous gene expression analysis.** Percentage of common DE genes in each *in planta* condition (versus rich medium) compared to previous *in planta* gene expression analyses (- Puigvert *et al*. 2017; -Jacobs *et al*. 2012; -Khokhani *et al*. 2017). Fractions represent the overlapping genes from the total of DEGs in each of our conditions compared to a given previous gene expression analysis. Colours were plotted using the Conditional Formatting tool in Microsoft Excel.



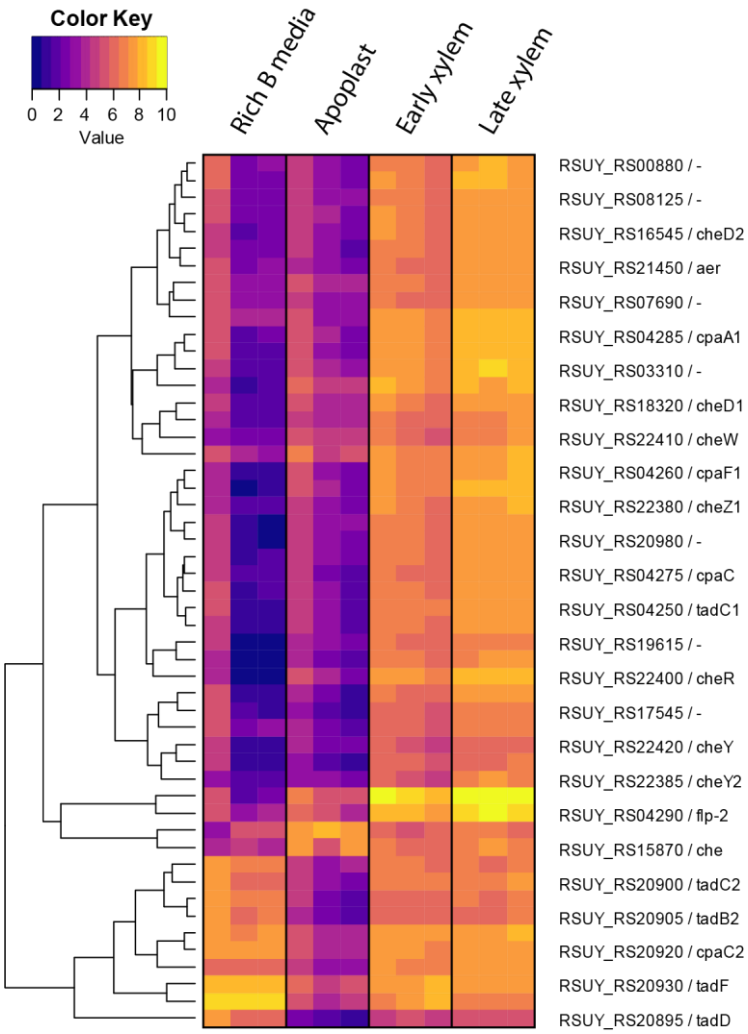
Additional File 7. **Transcriptomic profile of *R. solanacearum* in *in planta* environments**. Shared and unique DE genes across the three *in planta* conditions for the up-regulated (left) and down-regulated (right) genes. Each vertical bar plot represents the number of shared DE between the conditions indicated by the lines and dots in the schematic below. The horizontal bar plots on the right indicate the total DE genes per *in planta* condition compared to rich medium. For the intersection of Apoplast, Early and Late (*in planta* environment), Early and Late (Xylem environment), Apoplast and Late xylem alone, the list of genes was extracted and surveyed for enriched GO terms. Dot plots of the enriched GO terms for the up- (left) and down-regulated (right) genes in each environment is shown below. DE genes were identified with DEseq2 (*p-adj* > 0.01, log2FC±1.5) and plotted using the R package UpsetR.



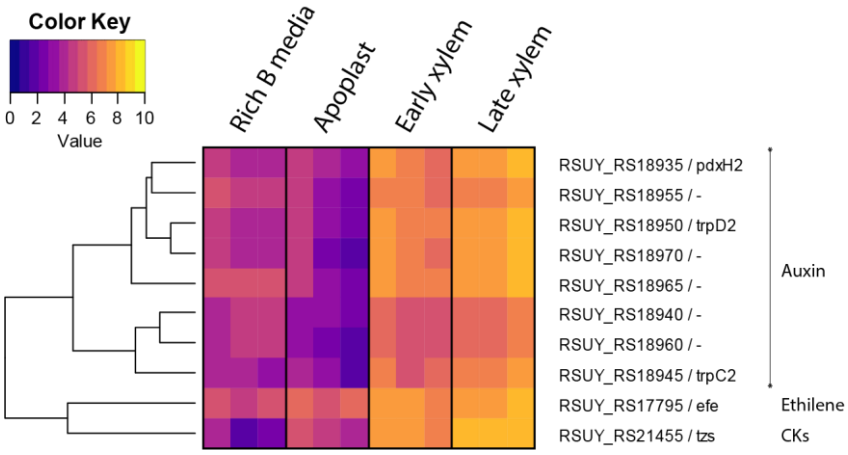
Additional File 8. **Gene expression dynamics of *R. solanacearum* throughout infection**. Six clusters were obtained through Mfuzz clustering of log2-fold-change data of the apoplast, early and late xylem conditions normalised to the reference rich liquid media. Clusters include the genes (number indicated above each graph) with a membership higher than 70% and consistently associated to the same cluster on at least 30 out of 40 iterations. The list of genes associated to each cluster was extracted and surveyed for enriched GO terms. Dot plots of the enriched GO terms in each cluster is shown next to the cluster.

https://drive.google.com/drive/folders/1E9W0H4Js0-CijGDVsQgTT3kxQF0kjaFV?usp=sharing

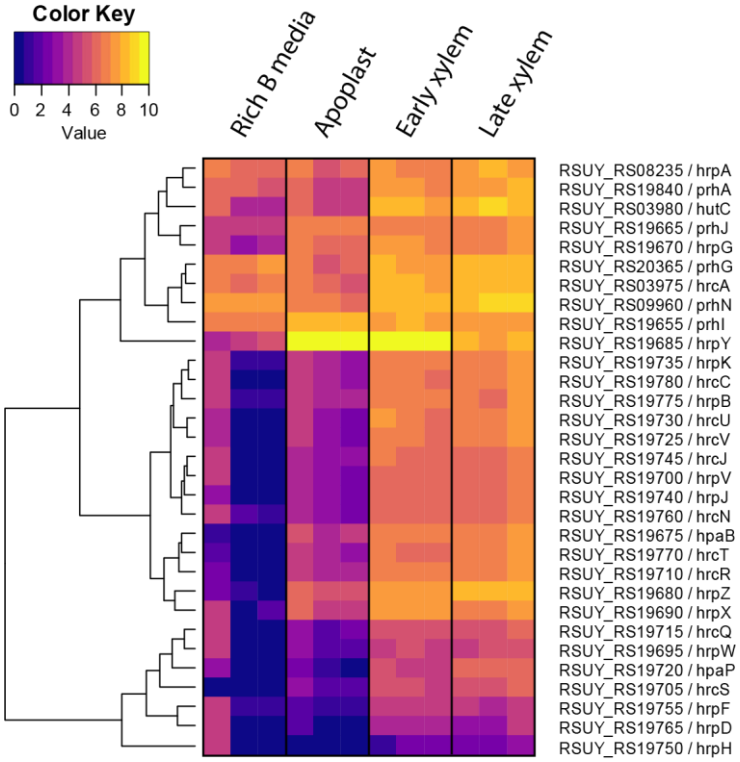
Additional File 9. **Transcripts Per Million of each gene in rich medium, apoplast, early and late xylem.** Reads normalized per Transcripts Per Million for each *R. solanacearum* gene in every condition: rich medium (philiq1, 2, 3), apoplast (Apo.10, .7, .9), early xylem (Early.D, .E, .G) and late xylem (Xylem.E, .O, Fresh.xylem). See **Note** (end of Chapter 1)



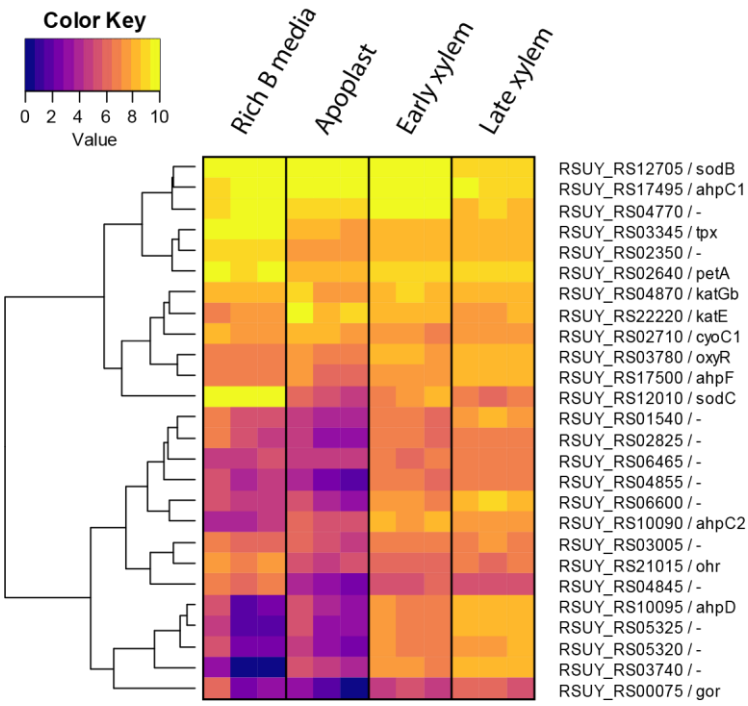
Additional File 10. **Chemosensors and signal transduction gene expression profile.** Heatmap showing the normalised transcripts per million (TPM) of the genes involved in chemosensoring and signal transduction in the reference condition and *in planta* apoplast, early and late condition.



Additional File 11. **Phytohormones biosynthesis gene expression profile**. Heatmap showing the normalised transcripts per million (TPM) of the genes involved in phytohormones biosynthesis in the reference condition and *in planta* apoplast, early and late condition.



Additional File 12. **T3SS regulatory cascade and apparatus gene expression profile.** Heatmap showing the normalised transcripts per million (TPM) of the genes involved in the T3SS regulatory cascade and the T3SS apparatus in the reference condition and *in planta* apoplast, early and late condition.



Additional File 13. **ROS scavenging enzymes gene expression profile.** Heatmap showing the normalised transcripts per million (TPM) of the genes coding for ROS scavenging enzymes in the reference condition and *in planta* apoplast, early and late condition.

Note: Additional Files 4, 5, and 9 can be accessed through the link on Google Drive inside the folder "Supplementary Figures - Dynamic expression of *Ralstonia solanacearum* virulence factors and metabolism-controlling genes during plant infection". This supplementary material is part of the publication under revision in BMC Genomics and will be available after published.

CHAPTER 2:

Characterization of *Solanum dulcamara* as a reservoir and overwintering host of *Ralstonia solanacearum*

Characterization of *Solanum dulcamara* as a reservoir and overwintering host of *Ralstonia solanacearum*

P. Sebastià[1], M. Valls[1,2*]

[1] Centre for Research in Agricultural Genomics (CSIC-IRTA-UAB-UB), Bellaterra, Catalonia, Spain
[2] Department of Genetics, University of Barcelona, Barcelona, Catalonia, Spain

*Correspondence: marcvalls@ub.edu

Abstract

Ralstonia solanacearum is the causal agent of bacterial wilt, a devastating plant disease responsible for serious economic losses especially on potato, tomato, and other solanaceous plant species in temperate countries. *R. solanacearum* phylotype II-B1 strains have caused important outbreaks in temperate countries in Europe in the last decades. The pathogen has been identified in *Solanum dulcamara* plants growing asymptomatically near rivers and potato fields. Thus, *S. dulcamara* has been described as a reservoir host where the bacterium can overwinter. However, the interaction between *R. solanacearum* and *S. dulcamara* and the traits that provide tolerance to this plant species remain uncharacterized. In our work, we have analysed the symptomatology, colonization patterns and the overwintering role of *S. dulcamara* compared to other susceptible hosts. We demonstrate that *S. dulcamara* behaves as tolerant to *R. solanacearum* thanks to its higher vasculature lignification, allowing massive bacterial multiplication but showing limited wilting symptoms. In addition, we showed that *S. dulcamara* tolerates *R. solanacearum* for long periods under cooler temperatures, suggesting its role as an overwintering host. This is the first report to provide a detailed description of the interaction between *R. solanacearum* and *S. dulcamara,* which will help broadening the knowledge about the pathogen's life cycle.

Introduction

A well-known concept in plant pathology is that a disease will be successfully established when there is a permissive environment, a virulent pathogen and a susceptible host (McNew, 1960), thus, changes in the environment will greatly influence the way plants and pathogens interact in nature. Although the interaction between a pathogen and its plant host is usually defined as susceptibility or resistance, there is a third type of interaction called tolerance. This concept has been ambiguously defined in the past, but a recent review defines tolerance as the ability of the plant host to, at similar pathogen loads, reduce the negative effects of the pathogen infection such as disease symptoms, yield reduction or host mortality rate. Extremely important in this definition is the fact that pathogen levels must be similar in the tolerant and the susceptible host, which breaks the model of disease severity that positively correlates the amount of pathogen within the host with disease symptoms (S Alizon et al., 2009; Samuel Alizon & Michalakis, 2015; Frank, 1996; Read, 1994). Tolerant hosts have shown its beneficial effects for the host survival, the pathogen life cycle and even for breeding for defence (Pagán & García-Arenal, 2020).

So-called alternate or reservoir host plants can asymptomatically host for long periods of time large amounts of pathogens, serving as inoculum source for further infections and intra and interspecific genetic rearrangements that fuel pathogen evolution (Haydon et al., 2002; Morris et al., 2009). Reservoir hosts have been extensively studied and identified for fungal pathogens, which use them to complete their sexual/asexual life cycles and to overwinter (Jie Zhao et al., 2016). For instance, *Puccinia graminis* f. sp. *tritici*, the causal agent of cereal rust, has a complex life cycle, performing its asexual phase in wheat and asymptomatically infecting its reservoir host *Berberis vulgaris* (common barberry) to complete its sexual phase (M. N. Wang et al., 2015). Bacterial plant pathogens also possess reservoir hosts. *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial blight in rice, can infect wild grasses of the genus *Leersia,* which is phylogenetically related to rice (Guo & Ge, 2005). Among them, different *Leersia* species have been identified as reservoir hosts of *X. oryzae* pv. *oryzae* such as *L. hexandra, L. sayanuka, L. oryzoides,* and *L. japonica* for their ability to host the pathogen without showing bacterial blight symptoms and serve as continuous source of inoculum to nearby rice plants (C. F. González, 1991; Lang et al., 2019). *Pseudomonas syringae* pv. *tomato*, the causal agent of bacterial speck in tomato, was shown to survive in seeds and asymptomatically infect weed plants near tomato field, which were used by the pathogen as sources of inoculum (McCarter, 1983). Interestingly, the complex water cycle of *P. syringae* has been widely analysed, and genetic and evolutionary studies sampling this pathogen from

different environmental reservoirs (e.g. waterways, snowpack) have underscored the importance of understanding the life of bacterial phytopathogens out of the crop host (Monteil et al., 2013). Similarly, *Xylella fastidiosa*, which causes Pierce's disease of grapevine, has been identified in several wild plants along permanent water sources (e.g. *Acer macrophyllum, Aesculus californica, Rubus ursinus*), suggesting a role of these reservoir hosts in the epidemiology and overwintering outside the grapevines (Hopkins, 1989; Purcell & Saunders, 1999). In a wider biological perspective, lichens have been identified as reservoir hosts of close relatives of important bacterial phytopathogens such as *P. syringae* (Liba et al., 2006) and *Burkholderia glathei* (Cardinale et al., 2006). However, not all bacterial plant pathogens use wild plant hosts to overwinter. For instance, the epiphytic *Erwinia amylovora*, is capable of surviving in a latent stage in infected stem cankers, waiting for better environmental conditions to multiply (Martins et al., 2018). All these studies show the importance of understanding the role of reservoir hosts in the epidemiology of bacterial plant pathogens to better understand their life cycles.

R. solanacearum has been shown to infect several wild plant species in the field outside its susceptible host. For instance, *R. solanacearum* phylotype II-B1 was identified in *Solanum nigrum* showing only wilting symptoms in those plants adjacent to potato wilting plants during a sampling experiment in Australia (Moffett & Hayward, 1980). *Solanum cinereum* (Graham & Lloyd, 1978) and stinging nettle (*Urtica dioica*) (Wenneker et al., 1999) were both shown to be perennial natural hosts of *R. solanacearum* phylotype II-B1 and it was demonstrated both under controlled and natural conditions that the infection was successful without any wilting symptom (Wenneker et al., 1999). A study carried out to identify new weed hosts of *R. solanacearum* from highland Uganda, found out that several wild plant species (e.g. *Amaranthus* spp., *Bidens pilosa, Galinsoga parviflora*) could be used as sources of inoculum to later on infect susceptible potato plants (Tusiime et al., 1997). Last but not least, *R. solanacearum* phylotype II-B1 strains have been also identified in the wild perennial plant *Solanum dulcamara* in different European countries in the last forty years (Elphinstone, 1996; Janse et al., 1998; Olsson, 1976).

S. dulcamara, commonly known as bittersweet, is a diploid (2n = 24), perennial weed, which belongs to the *Solanaceae* family (T. M. Golas, 2010). *S. dulcamara* is native from Europe but it has been naturalized in the entire holarctic area (Horvath et al., 1977), growing in contrasting environments (e.g. wet habitats, irrigation ditches, river banks) (T. M. Golas, 2010). Genetic analysis of a crossing population of different European *S. dulcamara* accessions identified one resistance gene (*Rpi-dlc1*) against *Phytophthora infestans,* the causal agent of potato late

blight (Tomasz Michal Golas et al., 2010). Interestingly, different reports have identified *S. dulcamara* as a wild host of *R. solanacearum*, proven that the pathogen has the capacity to survive and multiply in the roots of the weed asymptomatically (Elphinstone, 1996; Janse, 1996; Olsson, 1976). Following a chronological order, *R. solanacearum* phylotype II-B1 was sampled for the first time in Sweden in *Solanum dulcamara* plants growing next to wilting potato fields (Olsson, 1976). Later on, several reports have described *S. dulcamara* as a perennial wild host of *R. solanacearum* phylotype II-B1 in the Netherlands (Janse, 1996) and in England (Elphinstone, 1996). The fact that several outbreaks of *R. solanacearum* phylotype II-B1 strains were detected in different European countries, as well as the identification of *S. dulcamara* as a reservoir host, increases the interest to characterize the interaction between these two organisms.

Ralstonia solanacearum is a gram-negative β-proteobacterium, causing brown rot or bacterial wilt in more than 200 plant species, including economically important crops such as potato, tomato, peanut, eggplant and banana (Mansfield et al., 2012; Smith, 1896). Due to the large phenotypic and genotypic diversity, *R. solanacearum* is considered as a species complex (Prior & Fegan, 2005). Although the natural habitat of *R. solanacearum* are tropical and subtropical areas, phylotype II-B1 (traditionally named race 3 biovar 2) strains are adapted to cooler temperatures (Champoiseau et al., 2009), and have caused important outbreaks in traditionally pathogen-free temperate regions such as continental Europe (Elphinstone, 1996; Janse et al., 2004). *R. solanacearum* is a soil-borne pathogen, and enters the plant host through the root elongation zone, root wounds or secondary root emerging points (J. Vasse et al., 1995). Once inside the roots, the pathogen colonizes the apoplast while moving towards the plant vasculature (Yao & Allen, 2006). Crossing the Casparian strip, entering and colonizing extensively the xylem vessels is a hallmark of a successful *R. solanacearum* infection (Du et al., 2015; Hikichi et al., 2007), which finally leads to the production of large amounts of exopolysaccharide and clogging of the xylem, leading to wilting symptoms and plant death (Genin, 2010; J. Vasse et al., 1995). At this stage, *R. solanacearum* can return to the soil, where it can survive for long periods (Van Elsas et al., 2000), sometimes in a metabolically inactive viable but not culturable state (Steck, 2001). *R. solanacearum* survival has been also identified in waterways, which can serve as long-distance transport to infect new hosts elsewhere (Belén Álvarez et al., 2008).

R. solanacearum is a well-studied bacterial plant pathogen regarding its behaviour and interactions with susceptible crop hosts (e.g. potato, tomato). However, a thorough characterization of the interaction between the bacterial pathogen and its wild host *S.*

dulcamara is still pending. To understand better the interaction between *R. solanacearum* and *S. dulcamara*, we characterized different aspects of the symptomatology of this plant species, the localization of the pathogen during the infection process inside the plant, the mechanism by which *S. dulcamara* tolerates better the infection, and we tested the role of *S. dulcamara* as an overwintering shelter of *R. solanacearum*.

Materials & Methods

Plant and bacterial materials and growth conditions

The plants used in this project were: *Solanum dulcamara* plants coming from seeds harvested in (Vidrà, Girona, Spain), the susceptible tomato (*Solanum lycopersicum* cv. Marmande) (Leroy Merlin) and the susceptible potato (*Solanum tuberosum* cv. Desirée).

For pot experiments, *S. dulcamara* and *S. lycopersicum* cv. Marmande seeds were surface sterilized in 35% bleach and 0.02% Triton-X 100 for 10 minutes and then rinsed with sterile distilled water five times before sowing them on soil (Substrate 2, Klasmann-Deilmann GmbH) mixed with perlite and vermiculite (30:1:1) and grown in a chamber FITOCLIMA 1200 (Aralab) set at 22ºC with a LED lighting (60% humidity, 16 h day/ 8h night with a light intensity of 120-150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 3 weeks. To note: for optimal germination, *S. dulcamara* seeds needed a stratification period of between 2 and 3 weeks before growing at 22ºC. *S. tuberosum* cv. Desirée potato plants were propagated *in vitro* (Puigvert et al., 2017) and 2-week old apex were sown in the same soil mixture described above and grown in the same conditions for 3 weeks.

All infection assays were performed using *R. solanacearum* strain UY031 (phylotype IIB, sequevar 1) isolated from potato tubers in Uruguay (Siri et al., 2011) carrying either the synthetic *luxCDABE* operon or the GFPuv fluorescent gene both under the control of the *psbA* promoter (Monteiro, Solé, et al., 2012). Bacteria were routinely grown at 30ºC in rich B medium supplemented with 0.5% glucose (Monteiro, Solé, et al., 2012).

Plant inoculation and pathogenicity assays

For soil-soaking and petiole inoculation assays, plants were grown for 3-4 weeks. Soil-soaking inoculations were performed by pouring 40 ml of a 10^8 colony-forming units $(CFU) \cdot ml^{-1}$ (OD_{600} = 0.1) bacterial suspension on every plant pot without disturbing the roots. Infected plants were kept in a growth chamber set at 20ºC (cold experiment) or 27ºC and scored for wilting symptoms using a scale from 0 to 4: 0 = healthy plant with no wilt, 1 = 25% wilting, 2 = 50%, 3 = 75%, and 4 = 100% of the leaves wilted. Petiole-inoculation assays were performed by

inoculating twice 5 µl of a bacterial solution of 10^6 CFU·ml^{-1} (OD_{600} = 0.001) with a sterile 0.3×13 mm needle (30GX ½", BD Microlance, Becton Dickinson) and plants were kept in a growth chamber set at 27ºC and scored for wilting symptoms as described before.

Assessment of bacterial invasion

Invasion of *S. dulcamara*, Marmande or Desirée plants by *R. solanacearum* was assessed using the luminescent and fluorescent strains. Petiole-inoculated plants with the luminescent strain were sliced from one node below to one node above the inoculation point using a sterile razon blade. 1mm thick transversal cuts and 1 cm long longitudinal cuts were placed flat on a square plate and visualized using a live imaging system (ChemiDoc Touch Imaging System, Bio-Rad) using a 5-minute exposure time with 3×3 sensitivity. Images were processed using Image Lab software (Bio-Rad). Soil-soaking inoculated plants with the luminescent strain were photographed by placing the whole plant in a Fuji Film LAS4000 light imager system with a 15-minutes exposure time. To quantify the amount of bacteria inside the infected plant, 2 cm sections were cut from above the taproot (soil-soaked plants) or above the inoculation point (petiole-inoculated plants) and incubated for at least 30 minutes in a sterile 2 ml tube with 300 µl of sterile distilled water. Luminescence was measured with a luminometer (FB 12, Berthold Detection Systems). The relative light units per second (RLU·s^{-1}) were related to CFU·g^{-1} tissue after dilution plating of samples and CFU counting 24 h later.

Petiole-inoculated plants with the fluorescent strain were dissected as described before and photographed using binocular microscopy with a UV fluorescent lamp (BP330-385 BA420 filter) and a SZX16 stereomicroscope equipped with a DP71 camera system (Olympus) using the following settings: GFP filter, 10 seconds exposure time, ISO 1/800. Soil-soaked plants with the fluorescent strain were photographed with the upright microscope (Leica DM6) merging the UV channel for plant structures (blue) and the GFP channel for bacteria (green).

Tissue stainings

Soil-soaked plants with a bacterial solution of 10^7 CFU·g^{-1} (OD_{600} = 0.01) previously root-wounded with a 1 ml pipette tip, were transversally sliced in the taproot. 4-5 slices per plant were placed in a 1.5 ml tube with 70% ethanol for at least 7 days to remove the chlorophyll. For lignin staining, individual taproot slices were placed on a microscope slide incubating with two drops of phloroglucinol-HCl for about 1 minute, then rinsed with 70% ethanol and a cover slide was placed on top for visualization in the upright microscope (Leica DM6) bright field. Mock-infected plants were inoculated with water. Quantification of the amount of lignin was

performed by selecting the vascular area in Marmande and Desirée plants and compare it with the same area in *S. dulcamara* plants using ImageJ software. Conversion of the images to a grey scale (8-bit image) and then transforming the mean grey value. Quantifications from all plant species and all treatments were tested for statistical differences using ANOVA and Least Significant Differences Fisher test (Statgraphics software).

For suberin staining, individual *S. dulcamara,* tomato Marmande and potato Desirée taproot slices were placed on a well containing a Sudan IV solution for 5 minutes and then rinsed in another well with ethanol 70%. Clean slices were placed on a slide and visualized with the UV filter Leica DM6.

Statistical analysis

Statistical analyses were performed using Statgraphics software. All statistical tests are indicated in the respective figure legends.

Results

S. dulcamara roots are one of the major restriction points against *R. solanacearum* infection.

To analyse the symptomatology of *S. dulcamara* against *R. solanacearum,* two different inoculation methods were used. The first one consisted in soil-soaking the plants with a with 10^8 CFUs·ml^{-1} bacterial solution without wounding the roots. Wilting symptoms were tracked for 4 weeks. 12 days post inoculation (d.p.i.), all susceptible tomato Marmande plants were completely wilted, while *S. dulcamara* wilting symptoms had just started to appear (Figure 1A). By 28 d.p.i., only half of *S. dulcamara* plants had completely wilted, suggesting that *S. dulcamara* limits *R. solanacearum* entry through the roots, which delays the development of the disease (*p-value* < 0.0001; Figure 1A). Bacterial levels were also measured once a week for 4 weeks. At 12 d.p.i, when all Marmande plants were completely wilted, the amount of *R. solanacearum* in *S. dulcamara* was significantly lower ($5{\cdot}10^7$ CFUs·g^{-1}) than in Marmande plants (≈10^9 CFUs·g^{-1}) (Figure 1B). We found out that the minimal bacterial threshold to cause wilting in *S. dulcamara* was around $5{\cdot}10^7$ CFUs·g^{-1} (Supplementary Figure 1), similar to what was previously reported for susceptible Marmande tomato plants (Planas-Marquès et al., 2020). The second inoculation method used in our experiments was the direct introduction of *R. solanacearum* in the plant vasculature by applying a bacterial droplet to the wounds caused at base of the petiole after removal of a leaf, which skips the first root infection steps. Eight days

after petiole inoculation with 10^6 CFUs·ml^{-1} of *R. solanacearum*, all Marmande plants were completely wilted, whereas only half of *S. dulcamara* plants had wilted (*p-value* < 0.0001; Figure 1C). Opposite to what was observed in the soil-soaking experiment, all *S. dulcamara* plants died 2 weeks after petiole inoculation (Figure 1C). Quantification of bacterial loads in the stem over time showed that *S. dulcamara* could withstand similar bacterial amounts than tomato Marmande plants, although *R. solanacearum* levels were more heterogeneous in the former (Figure 1D). In sum, although *S. dulcamara* can delay the development of wilting symptoms when *R. solanacearum* is petiole-inoculated, highest tolerance was observed when the bacterium was inoculated mimicking natural infection through the roots.

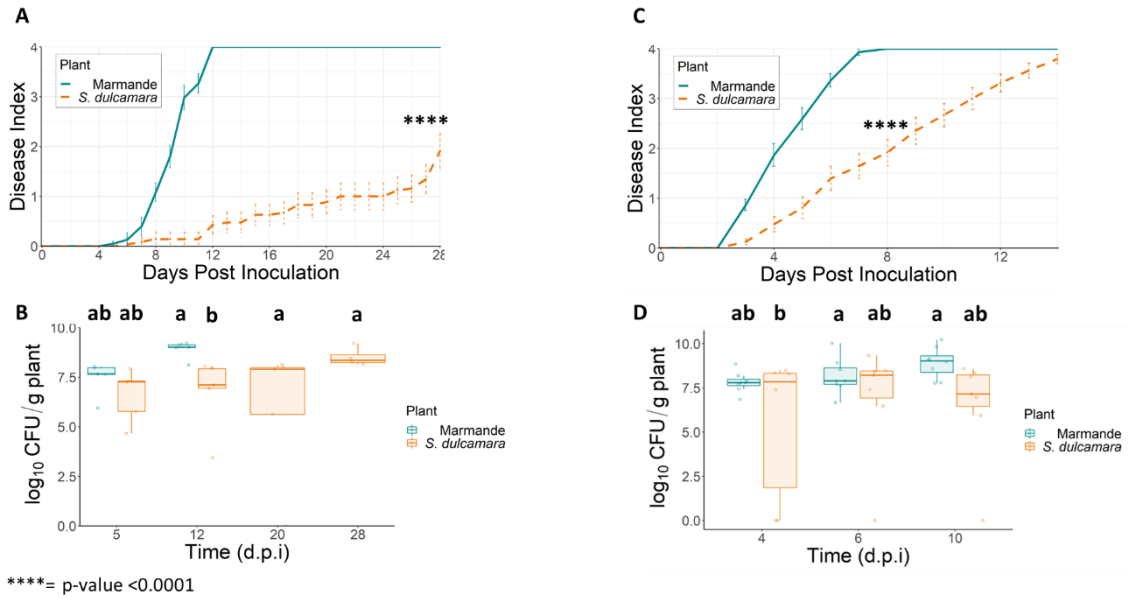


Figure 1. **Bacterial wilt symptomatology in soil- and stem- inoculated *S. dulcamara* and *S. lycopersicum* cv. Marmande plants.** *S. dulcamara* (orange) and Marmande (blue) plants were soil-soaked (A and B) or petiole-inoculated (C and D) with *R. solanacearum* UY031 carrying a luminescent-reporter. **A and C** show wilting symptoms over time with a scale from 0 (no wilting) to 4 (completely wilted). *n*= 30-35 plants per plant species **B and D** show bacterial levels in the stem from plants in A and C, respectively. Bacterial counts were calculated from tissue luminescence and are expressed as log CFUs·g^{-1} tissue. *n*= 4-8 plants per sampling day In **A. and C.** Different letters above each boxplot indicate statistically significant differences (α=0.05, Fisher's least significant difference test). **** indicate significant differences (*p value*<0.0001, T-student significant test α=0.05).

R. solanacearum colonizes *S. dulcamara* comparably to Marmande, without spreading to parenchyma cells.

After observing that *S. dulcamara* was capable of notably delaying the wilting symptoms and to have in general more tolerance than the susceptible host Marmande, we hypothesized that *S. dulcamara* restricts the spread of *R. solanacearum* to keep xylem vessels pathogen-free. To test this, we petiole-inoculated *S. dulcamara* and Marmande plants with a luminescent *R.*

solanacearum UY031 strain (Cruz et al., 2014). Live imaging of whole infected plants showed that *S. dulcamara* delays bacterial colonization at different disease stages (Figure 2A). In Marmande plants, *R. solanacearum* luminescence occupies the whole vasculature with high levels already at disease index 2, while in *S. dulcamara* the bacterial invasion is lower and parts of the upper stem remain free of luminescence, which indicates low pathogen levels (Figure 2A). Luminescence observation in longitudinal and transversal cuts of infected *S. dulcamara* and Marmande plants reinforced this result, although not as clearly as with the whole plant pictures (Supplementary Figure 2). Quantification of bacterial levels in *S. dulcamara* and Marmande plants at each disease stage showed significantly lower bacterial loads in *S. dulcamara* at disease index 0 (*p-value* < 0.05; Figure 2B). On the contrary, *S. dulcamara* held similar amounts of bacteria from disease index 1 to 3. Finally, the *R. solanacearum* counts at disease index 4 were again significantly lower (10^8 CFUs·g^{-1}) than in Marmande (10^{10} CFUs·g^{-1}) (*p-value* < 0.05; Figure 2B).

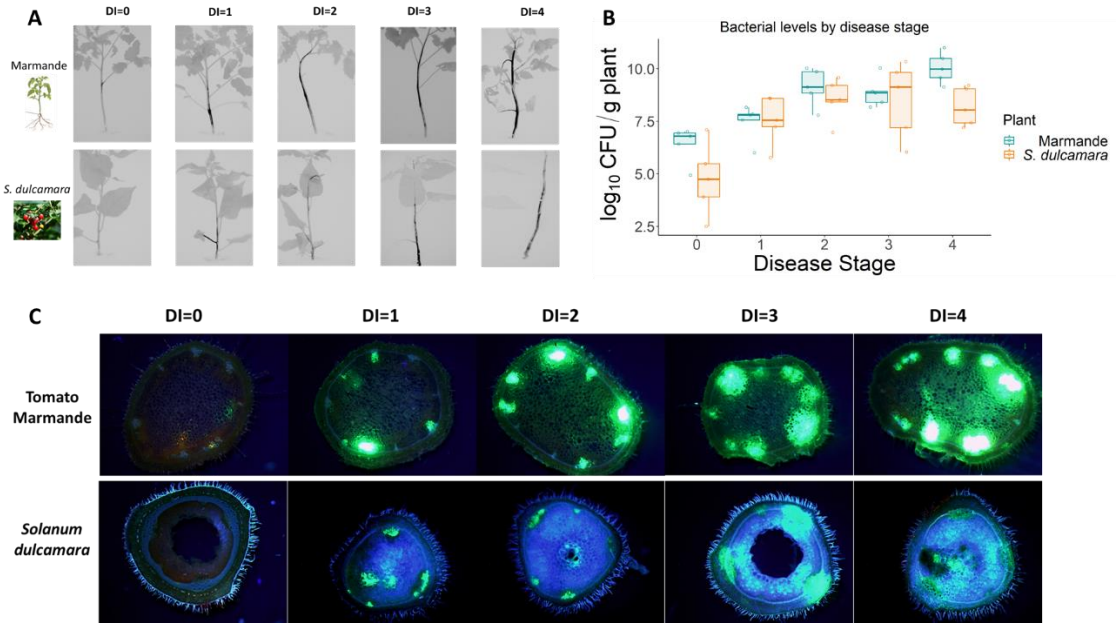


Figure 2. **Bacterial stem colonization and multiplication in stem-inoculated *S. dulcamara* and *S. lycopersicum* cv. Marmande plants**. **A.** Representative live imager photographs at different wilting stages (Disease index=0 to 4) of whole Marmande (top panel) and *S. dulcamara* (bottom panel) plants inoculated with luminescent *R. solanacearum*. **B.** Bacterial counts in the stem above the inoculation point from plants in (A) (*S. dulcamara* (orange) and Marmande (blue)) were calculated from the luminescence and are expressed as log CFU·g^{-1} tissue. *n*=5 plants per sampling day. * indicate significant differences (*p value*<0.05, T-student significant test α=0.05). **C.** Representative images at different wilting stages (Disease index=0 to 4) of internode of Marmande (top panel) and *S. dulcamara* (bottom panel) plants inoculated with GFP *R. solanacearum*.

To find out whether the bacterium was restricted to the xylem vessels in *S. dulcamara* or if it also colonized the surrounding parenchyma cells as observed in the susceptible tomato variety Marmande, we petiole-inoculated both plant species with a *R. solanacearum* UY031 fluorescent reporter strain (Cruz et al., 2014). Observation of internode cuts above the inoculation point at different disease stages showed that while bacterial colonization was delayed and restricted at the beginning of the infection in *S. dulcamara* (DI = 0-2), *R. solanacearum* was capable of extensive colonization of the whole xylem tissue at late infection stages in the reservoir host (DI = 3-4) (Figure 2C). On the contrary, although *R. solanacearum* could spread to neighbouring parenchyma cells in Marmande at mid and late infection stages (DI = 2-4), *S. dulcamara* restricted this movement, as indicated by fluorescence limited to the vessels (Figure 2C). An increase in UV-emission from the infected plant tissue was also apparent, suggesting an increase in phenolic compounds (Figure 2C lower panel). In summary, the assessment of bacterial colonization in both hosts showed a slight delay in bacterial levels and total colonization of the xylem vessels in *S. dulcamara*, which did not seem to fully explain the tolerant phenotype observed in the soil-soaking assays (Figure 1A).

S. dulcamara has a strong and stable lignified xylem structure.

Since observation of the colonization pattern of *R. solanacearum* in *S. dulcamara* compared to Marmande did not fully explain why *S. dulcamara* tolerates better bacterial wilt, we tested whether *S. dulcamara* showed pre-existing structures that enhanced defence. Lignin is one of the main components of the secondary plant cell wall, and it has been described to play an important role as a structural defence mechanism in resistant tomato varieties against *R. solanacearum* (Ishihara et al., 2012; Nakaho et al., 2000). Therefore, we tested whether *S. dulcamara* xylem vessels presented differential accumulation of lignin in their cell walls. Besides tomato cv Marmande, potato cv Desirée was also included in these experiments in order to assess our hypothesis in a cold adapted crop and widen the extent of our results. *S. dulcamara*, tomato cv Marmande and potato cv Desirée plants were soil-soaked with a 10^7 CFUs·ml^{-1} *R. solanacearum* solution to ensure a more homogeneous bacterial infection and only test for lignin differences between plant species. Tap root cuts obtained at 6 and 9 d.p.i. were stained with phloroglucinol HCl to stain lignified structures. Upon *R. solanacearum* infection, *S. dulcamara* xylem vessels and surrounding parenchyma cells showed a stable lignified structure (Figure 3A and 3B). On the contrary, both Marmande and Desirée plants showed a significant decrease in lignin accumulation both at 6 and 9 d.p.i. upon *R.*

solanacearum infection (Figure 3A and 3B). Moreover, the tap root vasculature structure in *S. dulcamara* is notably different than that one in Marmande and Desirée, showing already a massive lignification in the mock treatment both at 6 and 9 d.p.i., while in Marmande and potato, the parenchyma cells surrounding the vascular cylinder are not lignified at all (Figure 3A). In the lignin quantification (Figure 3B), the measured area for lignin quantification in *S. dulcamara* both in mock and infected treatment had always notably higher amounts of lignin stained compared to the susceptible hosts. To rule out the effect of other cell wall compounds in the restriction of *R. solanacearum* colonization in *S. dulcamara*, we tested whether there was an increase of suberin levels upon bacterial infection. However, we could not observe any accumulation of suberin either in the mock treatment nor upon infection in *S. dulcamara* as shown by Sudan IV staining, which should stain in a green to brown colour the suberised structures within the vascular cylinder (Supplementary Figure 3).

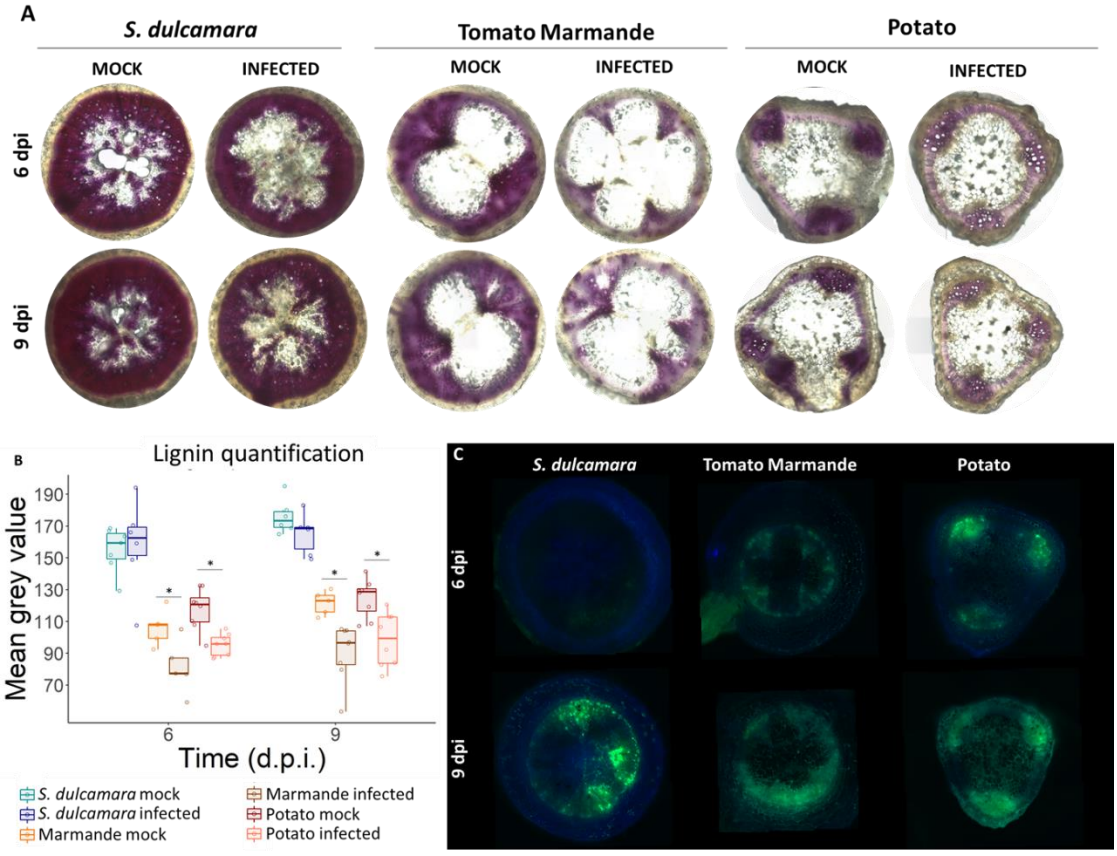


Figure 3. **Lignification of *S. dulcamara*, *S.* lycopersicum cv. Marmande and *S. tuberosum* cv. Desirée plants upon *R. solanacearum* infection. A.** Representative lignin staining images of taproot slices of *S. dulcamara* (leftmost), Marmande (centre) and potato (rightmost) plants mock and infected (6 and 9 d.p.i.). **B.** Lignin quantification of *S. dulcamara*, Marmande and potato upon mock and *R. solanacearum* infection (*p-value* < 0.05; T-student significant test α=0.05). **C.** Representative images of taproot slices at different infection times (6 and 9 d.p.i.) of *S. dulcamara* (leftmost), Marmande (centre) and potato (rightmost) plants inoculated with GFP *R. solanacearum*.

To find out whether the higher xylem vessel lignification in *S. dulcamara* could restrict *R. solanacearum* movement compared to Marmande and Desirée, we carefully observed the pattern of bacterial colonization by soil-soaking with a *R. solanacearum* GFP reporter strain at the same times after inoculation (6 and 9 d.p.i.) (Figure 3C). At 6 d.p.i., Marmande and Desirée plants showed their xylem vessels heavily infected while *S. dulcamara* only showed partial colonization (Figure 3C). At 9 d.p.i., Marmande and potato were completely colonized, including all the surrounding parenchyma cells, while *S. dulcamara* still had free xylem vessels to allow the upstream water flow (Figure 3C). In sum, *S. dulcamara* presented a pre-existing accumulation of lignin that it was not impaired upon pathogen infection in their xylem vessels and surrounding parenchyma that allowed a restriction of bacterial colonization compared to other susceptible hosts (i.e. Marmande and Desirée).

S. dulcamara survives bacterial wilt infection at cool temperatures.

While *R. solanacearum* could be detected in field soils no longer than two years after harvesting of potato crops wilted (Shamsuddin et al., 1978), several studies have shown detectable levels of *R. solanacearum* in aquatic roots of asymptomatic *S. dulcamara* plants, suggesting the reservoir host as a comfortable environment for *R. solanacearum* to survive winter conditions (Caruso, Palomo, Bertolini, Álvarez, et al., 2005; Elphinstone et al., 1998; Janse et al., 1998; Olsson, 1976; Wenneker et al., 1999). Accordingly, we tested whether the tolerant phenotype of *S. dulcamara* against *R. solanacearum* was even more significant at cooler temperatures, suggesting that *S. dulcamara* could serve as an overwintering shelter of *R. solanacearum.* We soil-soaked unwounded *S. dulcamara* and Desirée plants with 10^8 CFUs·ml^{-1} of *R. solanacearum* UY031 luminescent-reporter strain and tracked the survival rate and bacterial levels for 30 days at 20ºC, instead of 27ºC, which is the optimal growth temperature of *R. solanacearum* used for inoculation assays. Two weeks after infection, the first Desirée plants started to wilt, and by 30 d.p.i., around 50% of the Desirée plants were completely wilted (Figure 4A). On the contrary, all *S. dulcamara* plants survived the infection at 30 d.p.i. (Figure 4A), and only some plants showed individual wilted leaves, not reaching the disease index 0.5 (Supplementary Figure 5A). Quantification of bacterial levels at similar wilting stages in both *S. dulcamara* and Desirée, showed that the reservoir host could withstand comparable bacterial amounts in asymptomatic and early wilting plants (Figure 4B leftmost and centre). Since no *S. dulcamara* plant was completely wilted, bacterial levels at late wilting stage were only checked for Desirée plants (Figure 4B rightmost). After 30 days of infection at

20ºC, *R. solanacearum* counts in *S. dulcamara* reached about $5 \cdot 10^8$ CFUs·g^{-1}, while completely wilted Desirée plants harboured up to 10^{10} CFUs·g^{-1}. In conclusion, *S. dulcamara* could survive *R. solanacearum* infections at cooler temperatures without completely wilting, while keeping high amounts of bacteria inside the vasculature.

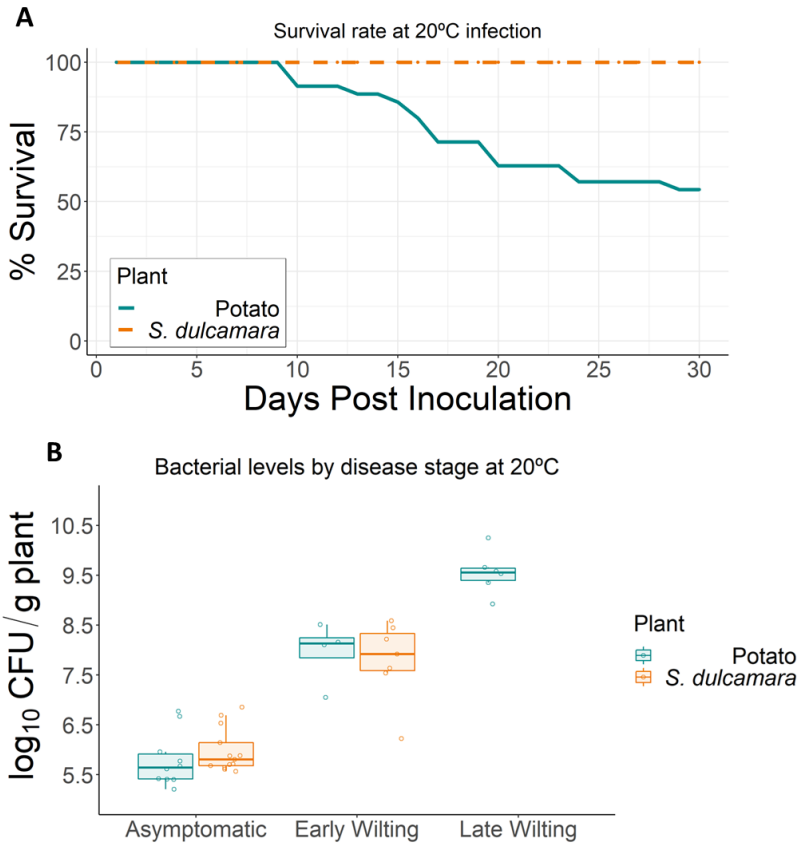


Figure 4. **Survival rate and bacterial multiplication in *S. dulcamara* and *S. tuberosum* cv. Desirée plants. A.** Survival rate of *S. dulcamara* (orange), potato (blue) plants inoculated with a *R. solanacearum* UY031 luminescent strain at 20ºC. *n* = 35 plants per species. **B.** Bacterial levels of asymptomatic (DI=0; rightmost), early wilting (DI=1-2; centre) and late wilting (DI=3-4; rightmost) were calculated from the luminescence from taproot sections of plants in (A) and are expressed as log CFU·g^{-1} tissue. *n* = 6-12 plants per sampling condition.

Discussion

R. solanacearum outbreaks in Europe in the last decades have increased the concern for spreading bacterial wilt disease in the continent (Elphinstone, 1996; Janse, 1996). Several reports identified the bacterial pathogen in aquatic roots of the native bittersweet (*S. dulcamara*) (Elphinstone, 1996; Olsson, 1976). Due to the lack of a precise characterization of the infection process of *R. solanacearum* in *S. dulcamara*, we decided to perform these inoculation assays in this so-called reservoir host.

The ability to keep high bacterial levels without showing symptoms is an interesting trait of reservoir hosts, providing an inoculum source of pathogen for the following cropping season (M. G. Roberts & Heesterbeek, 2020). According to this idea, we call reservoir hosts as tolerant hosts of their respective pathogens, since they allow pathogen multiplication without showing great damage to plant fitness (Pagán & García-Arenal, 2020). Although much research for sources of pathogen control has focused on resistance genes and thus limit pathogen's multiplication and imposing a selection pressure, tolerance offers a new strategy to deal with plant pathogens that should be incorporated into plant breeding programmes (Pagán & García-Arenal, 2020). We have used the concept tolerant *S. dulcamara* because it completely fitted the definition reviewed by Pagán and García-Arenal (Pagán & García-Arenal, 2020), according to which *S. dulcamara* reduces the negative effects of pathogen infection maintaining similar levels of *R. solanacearum* compared to the susceptible host. Contrary to what happens in the resistant tomato Hawaii 7996 where both *R. solanacearum* multiplication and symptoms are very low, *S. dulcamara* allows *R. solanacearum* to multiply with much less bacterial wilt damage in the reservoir host.

S. dulcamara delays bacterial wilting, especially when the more natural soil drench inoculation is used.

In our study, we have used both soil-soaking and petiole inoculation methods to dissect the symptomatology and interaction between *S. dulcamara* and *R. solanacearum*. The soil-soaking inoculation, mimicking a natural infection through the roots, showed that *S. dulcamara* presents a tolerant phenotype against *R. solanacearum* compared to the susceptible host Marmande (Figure 1A). This tolerance could be explained by differences in root architecture between *S. dulcamara* and other susceptible hosts, as already reported previously when comparing the resistant tomato variety Hawaii 7996 with other susceptible tomato varieties (D. Caldwell, 2016). Our lab previously showed that Hawaii 7996 restricts *R. solanacearum* colonization by four different means: root colonization, stem vertical movement, and circular and radial xylem colonization (Planas-Marquès et al., 2020). Thus, the tolerant phenotype shown by *S. dulcamara* after soil-soaking inoculation could be explained also by lower or delayed *R. solanacearum* root colonization (Figure 1A). Supporting this delayed wilting in *S. dulcamara*, bacterial levels through the infection process were never as high in the reservoir host as in Marmande (Figure 1B). According to the bacterial quantification, we observed that the minimum bacterial threshold to cause wilting was around $5 \cdot 10^7$ CFUs$\cdot g^{-1}$ (Supplementary Figure 1), similar to what was previously shown for Marmande (Planas-Marquès et al., 2020). Tolerance of *S. dulcamara* has been also reported towards the potato late blight agent

Phytophthora infestans. Some *S. dulcamara* genotypes showed an overcompensation phenotype of larger stem size and increased biomass upon *P. infestans* infection (Masini et al., 2019). It would be interesting to study if any of these measured traits could be also overcompensated in *S. dulcamara* upon *R. solanacearum* infection as a tolerance mechanism.

To investigate if this tolerance was maintained during the stem xylem colonization, *R. solanacearum* was directly inoculated in the stem xylem vessels by petiole inoculation. Our results show that, by using a more aggressive inoculation method (i.e. petiole-inoculation), *S. dulcamara* had a lower degree of tolerance compared to root inoculation, but the bacterial wilt was still delayed compared to Marmande (Figure 1C). Interestingly, by the end of the petiole-inoculation assay, all infected *S. dulcamara* plants died, suggesting that the tolerance presented by *S. dulcamara* in the stem xylem vessels is weaker than in the root (Figure 1). Related to the work by Planas-Marquès *et al*., our result indicates that *S. dulcamara* allows a vertical movement of *R. solanacearum* once it reaches the stem xylem vessels, hence providing a lower degree of resistance than Hawaii 7996.

R. solanacearum colonizes the xylem vasculature of *S. dulcamara* similar to Marmande.

Once the bacterium reaches the plant vasculature, the plant can still manage to block or delay bacterial spread either by reducing the vertical movement in the stem or the circular/radial movement between xylem vessels (Planas-Marquès et al., 2020). To test if *S. dulcamara* tolerant phenotype to *R. solanacearum* was caused by a restriction of bacterial movement, we petiole-inoculated the reservoir host with a luminescence-reporter strain (Cruz et al., 2014). General observation of the bacterial spread in *S. dulcamara* and Marmande whole plants showed that *R. solanacearum* displayed delayed colonization of *S. dulcamara* tissues compared to Marmande (Figure 2A). Observation of whole plants infected with the luminescent-reporter strain at different disease stages showed that although the general colonization was similar in both hosts, *R. solanacearum* was able to reach upper parts of the stem in Marmande and to heavily colonize the entire plant, while in *S. dulcamara* the upper part was kept free from the pathogen and at late wilting stages the stem was not that heavily colonized (Figure 2A). These colonization differences could be explained by the fact that *S. dulcamara* restricts *R. solanacearum* to specific xylem vessels, while others remain pathogen-free, as already reported for the resistant cv. Hawaii 7996 (Planas-Marquès et al., 2020).

Bacterial quantification at the very beginning of the infection as significantly lower in *S. dulcamara,* explaining the delay in bacterial wilt (Figure 2B). In addition to that, the amount of *R. solanacearum* inside *S. dulcamara* at very late wilting was significantly lower than in Marmande (Figure 2B). Bacterial counts in *S. dulcamara* together with the minimum bacterial threshold for causing bacterial wilt (Supplementary Figure 1) tell us that this plant species is very tolerant to *R. solanacearum* at low and intermediate bacterial loads, while at high bacterial levels this tolerance decreases, and bacterial wilt symptoms appear. Several studies have described tolerance as the reduction in the host health fitness maintaining stable pathogen loads in contrary to susceptibility, where high bacterial loads cause an extensive disease development (Pagán & García-Arenal, 2020; Råberg, 2014). According to these investigations, *S. dulcamara* presents an intermediate tolerant phenotype against *R. solanacearum*, since host fitness is maintained until a certain bacterial load and then bacterial wilt symptoms start to appear as *R. solanacearum* levels increase.

To take a closer look to the colonization process, we infected *S. dulcamara* and Marmande plants with a *R. solanacearum* GFP-reporter strain (Cruz et al., 2014). Stem slices observation showed that *S. dulcamara* had a different xylem and surrounding parenchyma cell structure compared to Marmande, containing a higher amount of lignin in the reservoir host (Figure 2C). This fact could be responsible for a lower infection level and restriction in specific xylem vessels during early infection stages (DI = 1-2) and a decrease in neighbouring parenchyma cells invasion in *S. dulcamara* compared to Marmande (DI = 3-4) (Figure 2C). Restriction of circular movement has been a conserved mechanisms for resistance against different bacterial vascular pathogens such as tomato (D. Caldwell et al., 2017) and Desirée (Cruz et al., 2014) against *R. solanacearum* and grapevine against *Xylella fastidiosa* (Chatterjee, Newman, et al., 2008). In addition to that, restriction of bacterial colonization to primary xylem vessels while the plant keeps the secondary xylem vessels still functional (Esau, 1977) could explain why *S. dulcamara* survives *R. solanacearum* infection while withstanding high amounts of bacteria inside.

Constitutive xylem vessel lignification in *S. dulcamara* supports its tolerant phenotype against *R. solanacearum.*

As previously reported for tomato resistance (Planas-Marquès et al., 2020), *S. dulcamara* is preventing *R. solanacearum* colonization by restricting its circular and horizontal movement across the xylem vessels. This phenomenon is clearly shown in Figure 3C, where GFP-reporter *R. solanacearum* infected all the vasculature of Marmande and potato plants at 6 d.p.i. and

further spread to parenchyma cells at 9 d.p.i., while in *S. dulcamara* the bacterial colonization was scarce at 6 d.p.i. and by 9 d.p.i. there were still xylem vessels without pathogen and very little parenchyma colonization. The fact that *S. dulcamara* showed a tolerant phenotype to *R. solanacearum* compared to Marmande was still puzzling since we could not explain which mechanism *S. dulcamara* was using to restrict the bacterial movement and delay wilting symptoms. *S. dulcamara* is a perennial shrub, whose appearance one month old is of a lignified plant. Lignification of xylem vessels (Ishihara et al., 2012) and xylem organization (D. Caldwell, 2016) have been shown to be important in restricting the colonization of *R. solanacearum* to primary xylem vessels and prevent the spread to secondary xylem vessels. Our lignin staining showed that while Marmande and Desirée tap roots reduce their lignin content significantly both at 6 and 9 d.p.i upon *R. solanacearum* infection, *S. dulcamara* plants with comparable bacterial levels, maintained their strongly lignified structures both in the xylem and neighbouring parenchyma cells (Figure 3A). Interestingly, *S. dulcamara* presents this lignification before pathogen infection since mock-inoculated plants did not have significantly different lignin content than pathogen-inoculated ones (Figure 3B). According to this fact, Ishihara *et al*. found that lignin biosynthesis genes were upregulated in the bacterial wilt resistant tomato variety LS-89 upon *R. solanacearum* infection, supporting the role of this compounds in bacterial wilt resistance (Ishihara et al., 2012). In summary, *S. dulcamara* strong pre-existing lignified xylem vessels are likely the factor that supports reservoir host tolerance to *R. solanacearum*, although lignin-accumulation *S. dulcamara* mutants would be needed in order to fully confirm this hypothesis.

S. dulcamara has higher tolerance to _R. solanacearum_ at cooler temperatures.

The identification of *S. dulcamara* in the late 70s as an asymptomatic host of *R. solanacearum* raised the question about how the reservoir host and bacterium interact in the nature (Olsson, 1976). Our soil-soaking inoculation experiment at cooler temperatures (20ºC) with *R. solanacearum* UY031 cold-adapted strain revealed that *S. dulcamara* can harbour high bacterial loads for long periods asymptomatically, or at least, without completely wilting, supporting the tolerant phenotype of *S. dulcamara* (Figure 4). Desirée plants were used as susceptible hosts since it is a crop that is also adapted to cooler temperatures (Ingram & McCloud, 1984). Interestingly, previous research on phylotype II-B1 strains virulence in cooler temperatures showed that two weeks after infection, all tomato plants were wilted (Milling et al., 2009). In all our experiments, more than 50% of the Desirée plants died one month after inoculation (Figure 4A). This difference could be explained by the fact that our Desirée plants were 25 days old in the moment of inoculation, while Marmande plants used in Milling *et al.*

(Milling et al., 2009) were only 16 days old, presenting softer tissues and thus, easier to colonize by *R. solanacearum*. Moreover, our data indicates that *S. dulcamara* can withstand bacterial wilt and survive the infection at cooler temperatures without completely dying (Figure 4A), although some *S. dulcamara* plants showed disease index not higher than 1 three weeks post inoculation (Supplementary Figure 5). Soil-soaked *S. dulcamara* plants were also able to maintain bacterial populations similar to Desirée plants (Figure 4B).

The fact that we call *S. dulcamara* a tolerant reservoir host for *R. solanacearum* is because of its ecological context, where *S. dulcamara* plants can latently maintain bacterial populations. Thus, at cooler temperatures, which are the most likely environmental conditions that *R. solanacearum* and *S. dulcamara* encounter in the European winter, the disease is not successfully established in *S. dulcamara* since the environment is not permissive enough and the reservoir host can resist the infection (McNew, 1960; Scholthof, 2007). Interestingly, all past cases of identification of *R. solanacearum* inside *S. dulcamara* were performed in aquatic roots of the wild host (Elphinstone, 1996; Olsson, 1976), where water stream carrying *R. solanacearum* was the source of infection. Under those conditions, *R. solanacearum* reached only detectable levels in the infected water stream during the warm summer, from June to October, when bacterial loads exceeded 10^5 CFUs·ml^{-1} (Elphinstone et al., 1998). In that same study, one-month old *S. dulcamara* infected plants under controlled conditions only developed wilting symptoms when soil inoculated with more than 10^6 CFUs · g plant^{-1}, although the inoculation temperature is not described (Elphinstone et al., 1998). Accordingly, in our cool experiment, high inoculum levels (10^8 CFUs·ml^{-1}) were used to infect potato and *S. dulcamara* plants, which caused that some *S. dulcamara* plants showed early wilting symptoms (Supplementary Figure 5).

All this combined, indicates that although previous reports had identified *S. dulcamara* as an asymptomatic reservoir host of *R. solanacearum*, this could be explained only under natural conditions, where *S. dulcamara* plants are old enough to have lignified structures, and bacterial levels in streams are below the threshold to cause disease in the wild host.

Conclusions

In summary, we performed a thorough characterization between the bacterial wilt agent *R. solanacearum* and its reservoir host *S. dulcamara.* Bacterial inoculation assays showed that *S. dulcamara* has a tolerant phenotype towards *R. solanacearum* while harbouring comparable bacterial levels to the susceptible host. Increased lignification of the xylem cylinder independently of the infection in *S. dulcamara* is one of the factors that provides this

tolerance. Bacterial inoculation assays under cold conditions showed that *S. dulcamara* can survive bacterial wilt for long periods, providing a new clue in how *R. solanacearum* overwinters inside the reservoir host. This study provides a deep characterization of the *R. solanacearum-S. dulcamara* system, which will set the basis for a better understanding of how the pathogen overwinters.

Abbreviations

CFU: colony forming units

cv.: cultivar

DI: disease index

d.p.i.: days post inoculation

f. sp.: forma speciales

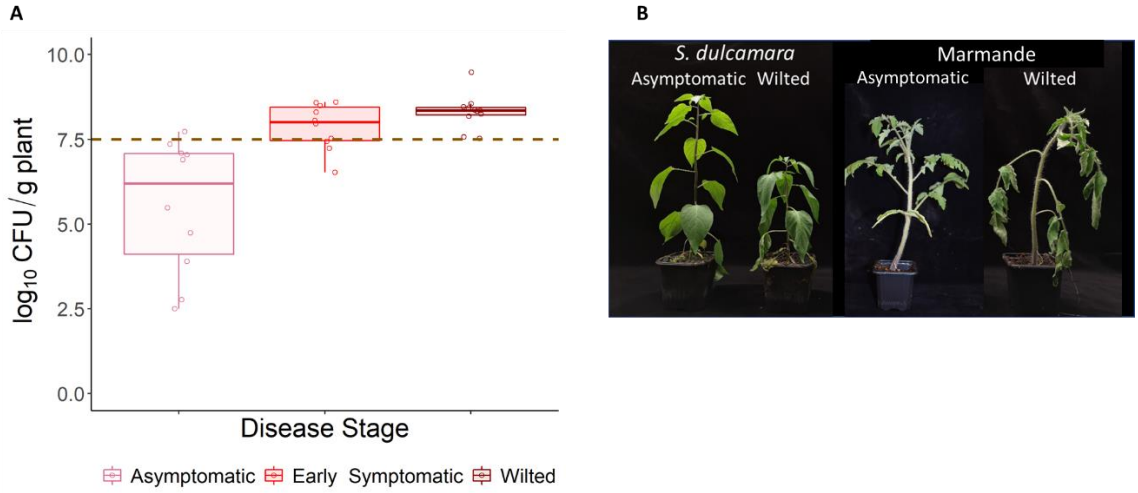
GFP: green fluorescent protein

OD: optical density

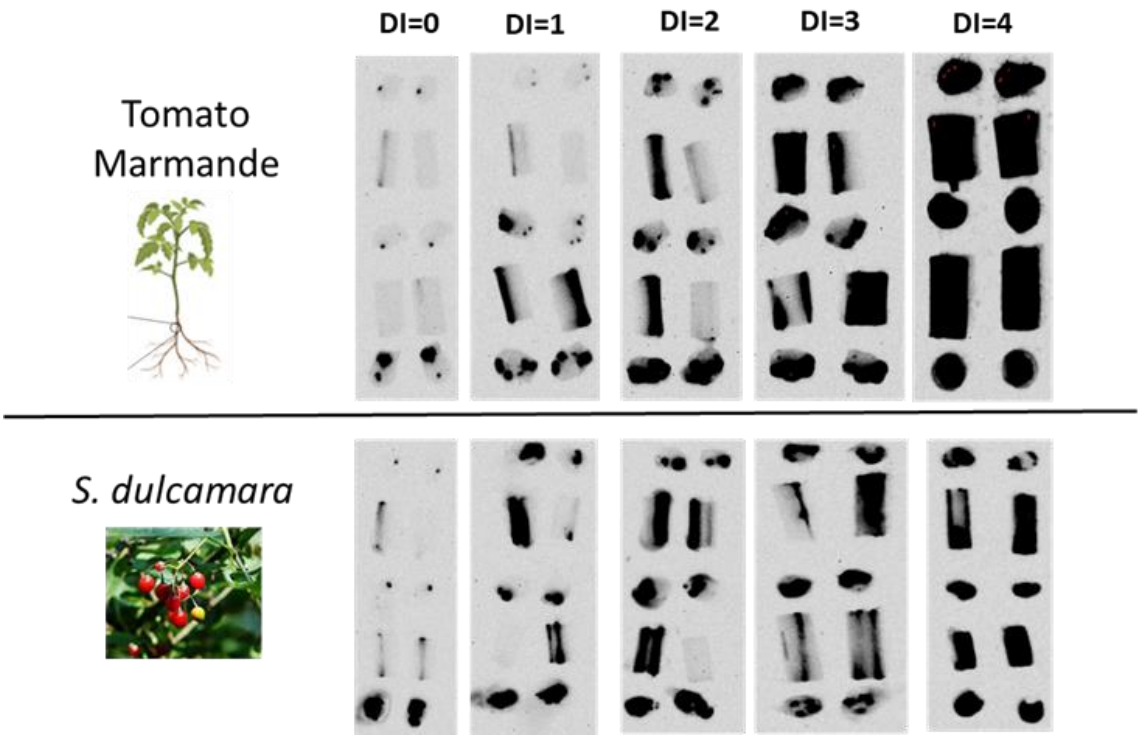
pv.: pathovar

RLU: relative light units

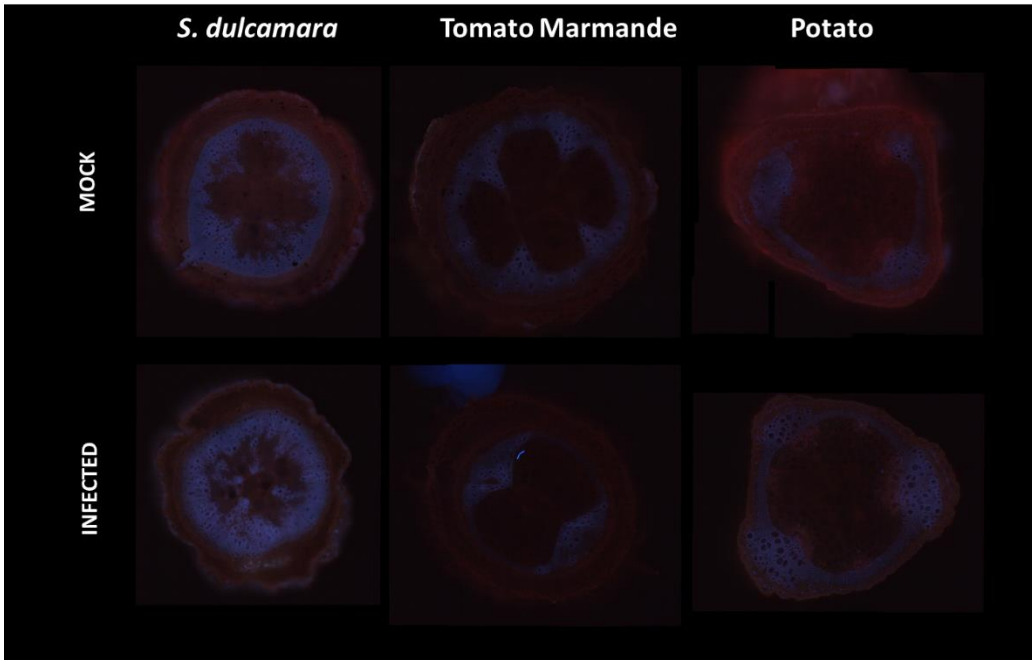
SUPPLEMENTARY MATERIAL



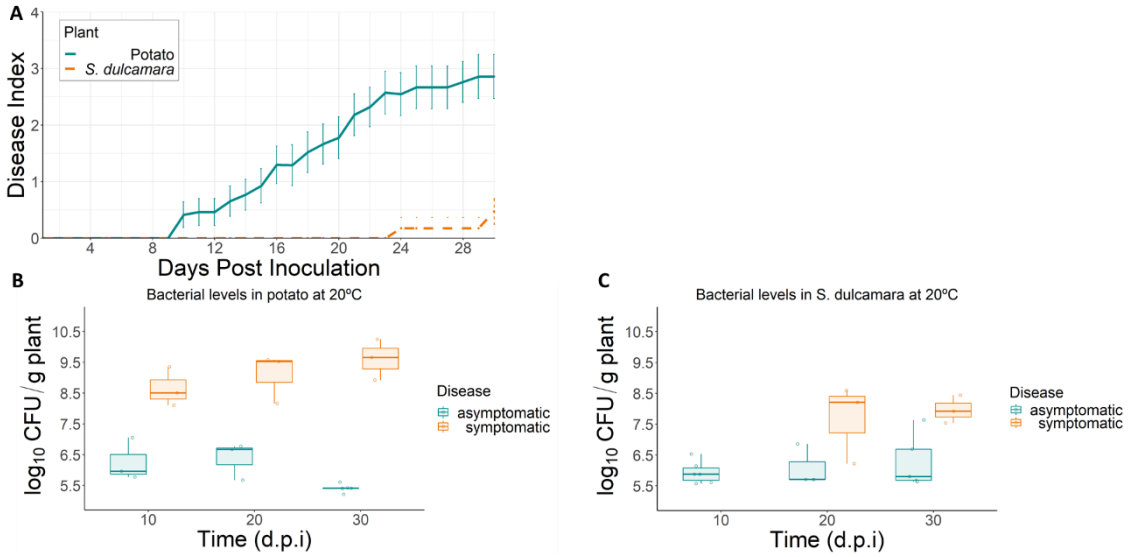
Supplementary Figure 1. **Minimum bacterial threshold to cause wilting in *S. dulcamara*. A.** Bacterial levels were quantified in asymptomatic, early wilting and completely wilted *S. dulcamara* plants inoculated with a *R. solanacearum* luminescent strain, and luminescence was used to calculate the log CFUs·g^{-1}. Horizontal brown dashed line indicates the separation between asymptomatic and early wilting plants. **B.** Pictures of asymptomatic/wilted *S. dulcamara* and Marmande plants.



Supplementary Figure 2. **Bacterial colonization of *S. dulcamara* vs *S.* lycopersicum cv. Marmande at different disease stages. A.** Representative photographs at different wilting stages (Disease index=0 to 4) of stem cuts of Marmande (top panel) and *S. dulcamara* (bottom panel) plants inoculated with luminescent *R. solanacearum*.



Supplementary Figure 3. **Suberin staining of *S. dulcamara*, *S.* lycopersicum cv. Marmande and *S. tuberosum* cv. Desirée.** Suberin observation in taproot slices mock and infected *S. dulcamara* (leftmost), Marmande (centre) and potato (rightmost) plants inoculated with a *R. solanacearum* UY031 luminescent strain.



Supplementary Figure 4. **Bacterial wilt and bacterial levels in *S. dulcamara* vs potato under cold conditions**. **A.** Bacterial wilting development (0=completely healthy to 4=completely wilted) in *S. dulcamara* (orange), potato (blue) plants inoculated with a *R. solanacearum* UY031 luminescent strain at 20ºC. *n* = 35 plants per species **B. and C.** Bacterial levels were quantified at different timepoints from asymptomatic (blue boxplots) or symptomatic (orange boxplots) potato and *S. dulcamara* respectively. Luminescence was used to calculate the log CFUs·g^{-1}. *n* = 3-6 plants per sampling day.

DISCUSSION

Thesis overview

In this work, we have characterized the life cycle of *R. solanacearum* phylotype II-B1 UY031 strain both along the infection in a susceptible host (Chapter 1), as well as the interaction with its reservoir host *S. dulcamara* (Chapter 2).

From *R. solanacearum in planta* dynamic gene expression to the rising saprophytic lifestyle question.

With the application of high throughput gene expression technologies in the study of *R. solanacearum*, several investigations focused their efforts in the understanding the behaviour of the bacterium in specific infection stages *in planta*. Thanks to these transcriptomic studies, different factors involved in plant infection were discovered such as genes associated with cool tolerance in phylotype II-B1 strains (Meng et al., 2015), genes that confer host specificity to *R. solanacearum* (Ailloud et al., 2016), the PhcA regulon *in planta* (Khokhani et al., 2017), the comparison of bacterial transcriptomes between infected susceptible and resistant potato roots (Puigvert et al., 2017) or the discovery of important bacterial metabolic adaptations to cope with the *in planta* environment (Dalsing et al., 2015; Jacobs et al., 2012). Except for the comparison of transcriptomes during potato root colonization, the rest of the transcriptomic studies were all performed sampling *R. solanacearum* from the xylem vessels at the onset of bacterial wilt, lacking a complete picture of the whole infection cycle in the plant host.

To fill this knowledge gap, we analysed in detail the gene expression dynamics of *R. solanacearum* along the infection process in potato plants (Chapter 1). To provide a more detailed description of *R. solanacearum* life inside the host, we sampled bacteria grown in three different plant infection conditions: leaf apoplast, early and late xylem colonization. Even though *R. solanacearum* is a soil-borne pathogen and colonizes the plant through the roots, there is no established protocol to achieve synchronized colonization of the root apoplast. Moreover, different reports have shown that *R. solanacearum* mutants lacking specific virulence factors have similar colonization patterns in the leaf and in the root (Hikichi et al., 2007; A. Kanda et al., 2009; Ayami Kanda et al., 2008; Ayami Kanda, Ohnishi, et al., 2003; Ayami Kanda, Yasukohchi, et al., 2003; Shinohara et al., 2005; Tsujimoto et al., 2008). Thus, we decided to use leaf apoplast as a mimicking condition for the early root apoplast colonization (Hikichi, 2016). Based on the differential gene expression analysis, we could group *R. solanacearum* behaviour into four different genetic programmes: all *in planta* conditions combined, apoplast, early and late xylem combined and late xylem (Chapter 1 Figure 1 and 2). Interestingly, we observed the strongest gene expression downregulation in the late xylem genetic programme, setting some interesting questions. Once *R. solanacearum* heavily

colonizes the xylem vessels of a susceptible host, it degrades secondary xylem vessels pit membranes and spreads to surrounding parenchyma cells (Planas-Marquès et al., 2020). The importance of this "out of the xylem" stage was recently underscored by a recent publication from our team, where it was shown that *R. solanacearum* can radially invade apoplastic cortical spaces at late infection stages in susceptible tomato Marmande plants (Planas-Marquès et al., 2020). In this new environment, the bacterium needs to adapt to survive in a decaying plant host. It is well known that *R. solanacearum* can survive for long periods in plant debris after the susceptible plant has died (Graham et al., 1979). However, little is known about which mechanisms is *R. solanacearum* deploying to successfully do it. In our transcriptomic study, we found out that *R. solanacearum* shuts down in the late xylem genes involved in carbon metabolism as well as the ribosomal machinery (Chapter 1 Figure 1). We took a closer look at the list of differentially downregulated genes in the late xylem but unfortunately, most genes are still unknown for their biological function and need a deeper characterization. We open an interesting window of study to decipher the gene regulation mechanisms, the virulence factors as well as the metabolic adaptations that the plant pathogen activates during its life once the plant host dies and the bacterium returns to the soil.

Complete gene expression landscape of *R. solanacearum* along the infection.

In Chapter 1, we carefully analysed the most relevant genes that *R. solanacearum* deploys to attack and adapt to the different plant environments along the infection. To identify which *R. solanacearum* gene categories were overrepresented in each condition, we performed an enrichment analysis of the KEGG and GO terms. However, KEGG enrichment analysis is limited to a number of preestablished pathways or terms that do not include important virulence categories, and because pathogenicity-related terms in GO are too general and have not been widely used, we decided to create a manually curated category that we defined as "virulence and parasitic fitness". This category included all genes encoding the type III secretion system (T3SS) and its associated effectors (T3Es), genes involved in motility, EPS and phytohormone biosynthesis, ROS scavenging genes, cell-wall degrading enzymes, and nitrogen metabolism (Chapter 1). Previous efforts have combined GO terms associated with plant-microbe interactions called PAMGO terms (Plant-Associated Microbe Gene Ontology) (Torto-Alalibo et al., 2009), including the term "pathogenesis" with associated subterms such as: interaction with host, adhesion to host, acquisition of nutrients from host, interaction with host via secreted substance, avoidance of host defences (Torto-Alalibo et al., 2009, 2010). The fact that these PAMGO terms have not been widely used in automatic GO term association studies, we

could not retrieve any enrichment of pathogenicity associated terms in our GO enrichment analysis in *R. solanacearum*, thus we decided to manually curate this "virulence and parasitic fitness" category which includes the most important *R. solanacearum* genes involved in virulence and adaptation to host (Chapter 1).

The first models of T3SS and T3Es gene expression indicated that they were repressed at high bacterial densities in culture medium (Genin et al., 2005; Yoshimochi et al., 2009). This discovery suggested that the T3SS and associated effectors would be important only at very early infection stages *in planta* when bacterial densities are not high and the *hrp/hrp* gene cluster would still be transcriptionally activated. However, further gene expression studies in plant hosts proved the contrary (Jacobs et al., 2012; Monteiro, Genin, et al., 2012), showing the importance of the T3SS along the infection and not just at early stages. According to these gene expression models, in Chapter 1 we demonstrated the activation of the *prh* cascade in the apoplast genetic programme leading to the subsequent induction of the HrpG and HrpB master regulators in the early xylem, followed by a maintained expression of most of the T3SS apparatus and T3Es genes in both early and late xylem conditions (Chapter 1, Figure 5). Interestingly, some T3Es had a different pattern of expression, such as *ripE2* being highly induced in the apoplast, *ripD* and *ripAD* were highly induced in all *in planta* infection stages and *ripI* with a remarkably low expression in all conditions (Chapter 1, Figure 3). The information available regarding the biological function of most T3Es is still scarce, thus our transcriptomic study shows a dynamic expression profile, which is a good starting point to investigate the biological relevance of these important bacterial proteins.

R. solanacearum thrives in diverse environments throughout the plant infection process. To successfully colonise its host, *R. solanacearum* needs to move through different media and surfaces such as the liquid root apoplast, the hollow xylem vessels, or the attachment to specific plant surfaces (Corral et al., 2020). As a general trend, our transcriptomic results confirmed previous motility studies in which swimming flagellar motility is highly induced in the plant apoplast (Tans-kersten et al., 2001), while type IV pili twitching motility is activated along the infection, with different bacterial functions, from attachment to root surface to adhesion and twitching inside the xylem vessels (Corral et al., 2020; Kang et al., 2002a) (Chapter 1, Figure 4). Specially puzzling was the fact that some flagellar motility genes (e.g. *motA, motB, fliC, fliD*), as well as some type IV pili genes (e.g. *fimT, pilE1, pilY1, pilW*) had low expression in the apoplast compared to the xylem environment. Detailed studies performed in the human pathogen *Pseudomonas aeruginosa* have described the role of each of the flagellar and type IV pili components (Sampedro et al., 2015), proving as helpful background for the

study of motility genes in *R. solanacearum*. Adapting the gene expression information that we provide in our dataset, together with the creation of *R. solanacearum* motility mutants will show the implication of each component in the swimming or twitching movement, as well as their role in virulence at specific infection stages, following the recent strategy of Corral *et al*. (Corral et al., 2020) in the identification of twitching regulatory genes and their implications in bacterial virulence *in planta*.

Metabolic adaptation is another key aspect in *R. solanacearum* genetic reprogramming to the plant environment. During the early xylem colonization, *R. solanacearum* rapidly consumes oxygen, providing high energy yields through oxidative respiration (Dalsing et al., 2015). Interestingly, *R. solanacearum* has the ability to use other molecules as terminal electron acceptors (TEA) such as nitrate, encoding the necessary genes for nitrate dissimilation, nitrate assimilation and denitrification (Dalsing et al., 2015). Recent studies showed the importance of nitrate, but not nitrite, as an energy source in the plant xylem in anaerobic conditions (Dalsing et al., 2015; Dalsing & Allen, 2014). In addition to that, *R. solanacearum* mutants lacking the nitrate reductase *narG* produced lower amounts of EPS, which impaired its virulence (Dalsing & Allen, 2014). In our transcriptome, we confirmed that nitrogen dissimilation, assimilation and denitrification genes were up-regulated in both early and late xylem (Chapter 1, Figure 6). Although at lower levels, these genes were also induced in the plant apoplast, which confirms previous reports indicating that *R. solanacearum* might have a background expression of nitrogen metabolism genes also in the apoplast, where *R. solanacearum* form microcolonies with low oxygen available, as shown for *Pseudomonas aeruginosa* human infections (Wessel et al., 2014). Upon pathogen attack, plants generate nitric oxide as a defence response against bacterial pathogens (Mur et al., 2017). *R. solanacearum* activates the expression of *hmpX*, a flavohemoglobin that can convert nitric oxide to nitrate, detoxifying the environment and preventing further bacterial damage (Dalsing et al., 2015). In our transcriptome, we also observed an up-regulation of *hmpX* in the early xylem, and maintained in the late xylem condition, indicating the importance of detoxifying the reactive nitrogen species produced by the plant as a defence mechanism as well as by the bacterial nitrate dissimilatory pathway (Chapter 1, Figure 6).

From the Greeen Revolution, high nitrogen inputs have been applied in the field, remarkably increasing crop yield. However, the overapplication of nitrogen fertilizers has been associated to pathogen proliferation in *Puccinia striiformis* (Neumann et al., 2004), and *P. syringae* pv. *tomato* (Olea et al., 2004), thus enhancing disease development (Mur et al., 2017). Although the amount of inorganic nitrogen is high enough in crop fields for promoting *R. solanacearum*

growth, it might not be the case in natural environments, where *R. solanacearum* needs to either survive in the soil and water or infect wild hosts without artificially applied nitrogen. Thus, it would be interesting to investigate the role nitrogen metabolism in all these ecological niches outside the susceptible host and how nitrate dissimilation promotes *R. solanacearum* growth in the anaerobic xylem environment.

S. dulcamara, a tolerant reservoir host of *R. solanacearum*

Reservoir hosts harbour a pathogen without getting the harmful effects of the infection and then show the ability to transmit the pathogen to the target host population (M. G. Roberts & Heesterbeek, 2020). All the reports that have identified *R. solanacearum* inside *S. dulcamara* have demonstrated to not cause wilting symptoms under natural conditions while being able to transmit *R. solanacearum* to nearby water streams and crop fields where the bacterium could infect new susceptible hosts (Elphinstone, 1996; Janse et al., 1998; Olsson, 1976). This observation underscores the importance of a reservoir host to tolerate the pathogen, thus a reservoir host needs *per se* to be able to tolerate pathogen multiplication without showing disease symptoms. This tolerant phenotype of *S. dulcamara* pinpoints a co-evolution of *R. solanacearum-S. dulcamara* towards an equilibrium in which the reservoir host is not harmed by the pathogen and *R. solanacearum* can survive inside *S. dulcamara* and spread around. From the pathogen perspective, reservoir hosts might be also ecological niches where genetic exchange happens intra and interspecifically, as discussed for the rice blast disease fungus *Magnaporte oryzae* (Langner et al., 2018). Thus, understanding how *R. solanacearum* behaves inside its reservoir hosts such as *S. dulcamara* will provide a broader ecological picture of the bacterial wilt pathogen life cycle.

The two main plant defence strategies to cope with pathogens are resistance (i.e. the plant ability to limit pathogen multiplication) (Clarke, 1986), and tolerance (i.e. the plant ability to reduce the negative effects of infection without limiting pathogen multiplication) (Pagán & García-Arenal, 2020). On one hand, resistance to *R. solanacearum* has been identified in some potato wild relatives such as *Solanum commersonii* (M. González et al., 2013), in the tomato variety Hawaii 7996 (Rivard & Louws, 2008), tobacco (Qian et al., 2013) and eggplant (Lebeau et al., 2013). In most cases this resistance is a polygenic trait, being extremely challenging to transfer it to a commercial variety without the introduction of linked undesirable characteristics (T. Denny, 2006). In addition, resistance reduces pathogen's fitness, imposing a selection pressure that might eventually lead to the resistance breaking down (Frantzen, 2007).

On the other hand, plants show different mechanisms to tolerate pathogen infections without showing disease symptoms. Increase in photosynthetic activity in non-infected leaves has been reported in cereal crops that tolerate different fungi (Ney et al., 2013). Other plants tolerate foliar and root pathogens by reallocating their resources between plant organs (Ayres et al., 2017; Rooney & Hoad, 1989). Tolerance to *Cucumber mosaic virus* in the model plant *Arabidopsis thaliana* has been associated with resource reallocation from growth to reproductive organs (Montes et al., 2019, 2020). *A. thaliana* tolerance to the bacterial pathogen *P. syringae* with increased biosynthesis of salicylic acid (Jian Zhao et al., 2013). In general, tolerance mechanisms differ from resistance mechanisms as the first ones involve plant strategies to cope with the pathogen infection without compromising plant fitness and crop yield, while resistance is associated with an inhibition of pathogen growth. In *R. solanacearum*, tolerant hosts have been reported mostly during the assessment of resistant/susceptible potato germplasm in different locations such as the Cruza potato cultivar from Kenya (Uwamahoro et al., 2020), different potato clones along with *Solanum sucrense* and *Solanum juzepczuki* (Chakrabarti et al., 1993), and several Irish potato cultivars grown in different African countries (Felix et al., 2010). Another study identified *R. solanacearum* tolerant crops as those ones invaded by high densities in xylem vessels in the root while the invasion of xylem vessels at mid stem is limited. Different crop species were reported as tolerant such as cabbage cultivars, cauliflower, kidney bean (B. Álvarez et al., 2008). Last but not least, another study considered as tolerant different tomato cultivars previously reported as resistant (i.e. CRA 66, Hawaii 7996 and Caraïbo) (Grimault & Prior, 1993). Interestingly, these tomato cultivars could harbour latent *R. solanacearum* infections and maintain some xylem vessels free from bacteria, allowing the upstream water flow and avoiding the appearance of bacterial wilt symptoms. Thus, the capacity of these plants of restricting *R. solanacearum* invasion to individual xylem vessels was considered as a hallmark of tolerance to bacterial wilt, apart from the ability to remain symptomless (Grimault & Prior, 1993). In all these cases, plant material was considered as tolerant when high bacterial counts were found while the effects of bacterial wilt were less severe than in other susceptible hosts. In summary, tolerance does not impose this selection pressure, thus providing a much stable and durable defence strategy in the field (R. M. Caldwell et al., 1958; Van Den Bosch et al., 2006).

In our symptomatology experiments, we traced bacterial wilt development as well as bacterial levels in *S. dulcamara* and tomato Marmande plants (Chapter 2, Figure 1). Interestingly, while we observed a reduction in wilting in *S. dulcamara* plants, the bacterial counts were similar to those sampled from Marmande plants. Our results indicate that *S. dulcamara* behaves as a

tolerant host of *R. solanacearum* since it shows the capacity to harbour high bacterial densities while delaying the appearance of wilting symptoms. The tolerance in the *S. dulcamara-R. solanacearum* interaction was higher when the pathogen was soil-inoculated, but this effect was milder when *R. solanacearum* was directly inoculated inside the xylem vessels, causing larger wilting symptoms in the reservoir host (Chapter 2, Figure 1). In order to identify the mechanisms that *S. dulcamara* uses to restrict *R. solanacearum* colonization, we hypothesized that the reservoir host might present a lignified structure that would keep some xylem vessels free from bacteria. Lignified vasculature is one of the quantitative traits associated to *R. solanacearum* resistance in the tomato cultivar LS-89 (Ishihara et al., 2012). Accordingly, we also observed a different vascular structure and higher lignin accumulation in *S. dulcamara* taproot compared to susceptible tomato and potato plants (Chapter 2, Figure 3).

Based on these results, we conclude that *S. dulcamara* behaves as a tolerant host of *R. solanacearum* because it can maintain high bacterial populations without showing wilting symptoms. At the same time *S. dulcamara* can be considered a reservoir host of *R. solanacearum* in its ecological context, since it has the ability to maintain high *R. solanacearum* densities for long periods and serve as a continuous source of inoculum for infections of susceptible hosts. Therefore, we state that *S. dulcamara* should be considered not just as a reservoir host of *R. solanacearum* which was already known, but also a tolerant host of the bacterial wilt pathogen. Our work sets a path for the study of *S. dulcamara* traits that provide tolerance to *R. solanacearum,* although a deeper characterization of the root system and its associated rhizosphere will be necessary to decipher the tolerance mechanism of this reservoir host.

Temperature influence on *R. solanacearum-S. dulcamara* interactions

R. solanacearum has a complex life cycle, and once the susceptible host dies from wilting, the bacterium returns to the soil and water (Belén Álvarez et al., 2008; Van Elsas et al., 2000), where it can survive in a metabolically inactive state called viable but not culturable state (VBNC) (Grey & Steck, 2001). Several reports have identified *R. solanacearum* phylotype II-B1 cold-adapted strains in *S. dulcamara* roots in river edges and next to potato fields in different European countries, suggesting that some wild plant species such as *S. dulcamara* could serve as overwintering shelter of the bacterial pathogen in temperate regions (Elphinstone, 1996; Hayward, 1991; Janse et al., 1998; Olsson, 1976). Demonstrating that *S. dulcamara* can withstand large amounts of *R. solanacearum* for long periods under cooler temperatures in

controlled conditions was a milestone that was missing towards the complete understanding of *R. solanacearum* life cycle.

In Chapter 2, we proved that *S. dulcamara* survives bacterial wilt infection for long periods in cooler temperatures compared to potato plants that died under the same conditions (Chapter 2, Figure 4). In our experiment, we inoculated three-week old potato and *S. dulcamara* plants with high bacterial inoculum and kept them at 20ºC in order to establish conditions in which *R. solanacearum* could still kill potato plants while *S. dulcamara* would still be asymptomatic. The results obtained under these conditions helped us to extrapolate the way *R. solanacearum* and *S. dulcamara* interact in nature. We used a remarkably high bacterial inoculum compared to the bacterial concentrations that are present in soil or waterways (Van Elsas et al., 2000), which could explain why some *S. dulcamara* still showed some wilted leaves instead of being completely asymptomatic as reported in previous studies (Elphinstone, 1996; Olsson, 1976). This remains an experimental limitation since inoculating with the bacterial levels observed in nature -10^2 CFUs·g^{-1} in soil (Van Elsas et al., 2000) and 10^4-10^6 CFUs·ml^{-1} in water (Belén Álvarez et al., 2008)- would result in extremely slow and heterogeneous infections. In addition, while *S. dulcamara* plants used in our experiments were 3-4 weeks old and thus not completely lignified, *S. dulcamara* is a perennial plant that in the nature presents a lignified structure and presumably this will make it even more tolerant to *R. solanacearum* infections than the phenotype that we observed in our experimental conditions. In plant-pathogen interactions, the environment is of crucial importance when determining the success of a pathogen infection (Francl, 2001). Under cooler temperatures, *R. solanacearum* multiplication rate would be much slower or even not multiplying at all given that its optimal growth temperature it is around 30ºC. Therefore, it would be expected that under a real temperate winter, low levels of *R. solanacearum* cold-adapted strains would infect perennial *S. dulcamara* resulting in detectable bacterial levels without causing wilting symptoms in the reservoir host. In conclusion, our results underscore how important the environment is in determining if an interaction between a host plant and a pathogen will result in a successful disease outcome. Temperature has shaped the interaction we observed between *R. solanacearum* and *S. dulcamara*, ranging from a mild tolerant phenotype at 27ºC where the wilting symptoms were just delayed compared to the susceptible Marmande plants, to a full tolerant phenotype without wilting symptoms at cooler temperatures (20ºC).

In summary, this work has contributed to the study of *R. solanacearum* life cycle. First, we have analysed for the first time the transcriptome of *R. solanacearum* along the infection process in potato plants. Gene expression pattern and enrichment of virulence factors have

provided a general picture of the bacterial adaptation to virulence and parasitic life inside the plant. Second, we have characterized the interaction between *R. solanacearum* and its main reservoir host in Europe: *S. dulcamara.*

CONCLUSIONS

From the main goals of this thesis we extract the following conclusions:

Characterization of the dynamic transcriptional profile of *Ralstonia solanacearum* in different infection stages in potato plants

1.1. Differential Gene Expression analysis of *R. solanacearum* in three different plant conditions (apoplast, early xylem and late xylem) compared to rich liquid medium, revealed that *R. solanacearum* deploys four specific gene expression programmes (i.e. *in planta*, apoplast, xylem, and late xylem) during infection.

1.2. *R. solanacearum* shows induction of flagellar-associated genes in the apoplast, while type IV pili-associated genes remain highly expression throughout the infection.

1.3. *R. solanacearum* expresses most of its type III effectors in the xylem environment.

1.4. *R. solanacearum* activates different nitrate respiration and detoxification genes in the xylem as a likely means to adapt to this hypoxic environment.

Characterization of *Solanum dulcamara* as a reservoir host of *Ralstonia solanacearum*

1.5. *S. dulcamara* shows a tolerant phenotype against *R. solanacearum*, delaying the appearance of wilting symptoms especially when inoculated through the roots and maintaining at the same time similar bacterial loads than the susceptible host Marmande.

1.6. *R. solanacearum* shows similar colonization patterns in *S. dulcamara* and Tomato cv. Marmande, which further supports the notion of *S. dulcamara* as a tolerant host, allowing bacterial movement without showing disease symptoms.

1.7. *S. dulcamara* displays a constitutively lignified vascular structure that is maintained upon *R. solanacearum* infection -contrary to what happens in susceptible tomato and potato-, which may partly explain the tolerant phenotype of *S. dulcamara*.

1.8. Under cooler temperatures, *S. dulcamara* shows higher tolerance against *R. solanacearum*, which explains its natural behaviour as a symptomless overwintering shelter of *R. solanacearum*

REFERENCES

Ailloud, F., Lowe, T., Cellier, G., Roche, D., Allen, C., & Prior, P. (2015). Comparative genomic analysis of Ralstonia solanacearum reveals candidate genes for host specificity. *BMC Genomics*, *16*(1), 1–11. https://doi.org/10.1186/s12864-015-1474-8

Ailloud, F., Lowe, T. M., Robène, I., Cruveiller, S., Allen, C., & Prior, P. (2016). In planta comparative transcriptomics of host-adapted strains of Ralstonia solanacearum. *PeerJ*, *2016*(1). https://doi.org/10.7717/peerj.1549

Alizon, S, Hurford, A., Mideo, N., & Baalen, M. V. A. N. (2009). *Virulence evolution and the trade-off hypothesis : history , current state of affairs and the future*. *22*, 245–259. https://doi.org/10.1111/j.1420-9101.2008.01658.x

Alizon, Samuel, & Michalakis, Y. (2015). Adaptive virulence evolution : the good old fitness-based approach. *Trends in Ecology & Evolution*, *30*(5), 248–254. https://doi.org/10.1016/j.tree.2015.02.009

Almeida, R. P. P., & Nunney, L. (2015). How do plant diseases caused by xylella fastidiosa emerge? *Plant Disease*, *99*(11), 1457–1467. https://doi.org/10.1094/PDIS-02-15-0159-FE

Álvarez, B., Vasse, J., Le-Courtois, V., Trigalet-Démery, D., López, M. M., & Trigalet, A. (2008). Comparative behavior of Ralstonia solanacearum biovar 2 in diverse plant species. *Phytopathology*, *98*(1), 59–68. https://doi.org/10.1094/PHYTO-98-1-0059

Álvarez, Belén, López, M. M., & Biosca, E. G. (2007). Influence of native microbiota on survival of Ralstonia solanacearum phylotype II in river water microcosms. *Applied and Environmental Microbiology*, *73*(22), 7210–7217. https://doi.org/10.1128/AEM.00960-07

Álvarez, Belén, López, M. M., & Biosca, E. G. (2008). Survival strategies and pathogenicity of Ralstonia solanacearum phylotype II subjected to prolonged starvation in environmental water microcosms. *Microbiology*, *154*(11), 3590–3598. https://doi.org/10.1099/mic.0.2008/019448-0

Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq-A Python framework to work with high-throughput sequencing data. *Bioinformatics*, *31*(2), 166–169. https://doi.org/10.1093/bioinformatics/btu638

Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R., & Daszak, P. (2004). Emerging infectious diseases of plants: Pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology and Evolution*, *19*(10), 535–544. https://doi.org/10.1016/j.tree.2004.07.021

Andrews, S., Krueger, F., Seconds-Pichon, A., Biggins, F., & Wingett, S. (2015). *FastQC. A quality control tool for high throughput sequence data. Babraham Bioinformatics*. Babraham Institute.

Arlat, M., Gough, C. L., Zischek, C., Barberis, P. A., Trigalet, A., & Boucher, C. A. (1992). Transcriptional organization and expression of the large hrp gene cluster of Pseudomonas solanacearum. In *Molecular plant-microbe interactions : MPMI* (Vol. 5, Issue 2, pp. 187–193). https://doi.org/10.1094/MPMI-5-187

Ayres, P. G., Press, M. C., & Spencer-Phillips, P. T. N. (2017). Effects of pathogens and parasitic plants on source-sink relationships. In *Photoassimilate Distribution Plants and Crops Source-Sink Relationships*. https://doi.org/10.1201/9780203743539

Baccari, C., & Lindow, S. E. (2011). Assessment of the process of movement of Xylella fastidiosa within susceptible and resistant grape cultivars. *Phytopathology*, *101*(1), 77–84. https://doi.org/10.1094/PHYTO-04-10-0104

Baker, R. H. A., Sansford, C. E., Jarvis, C. H., Cannon, R. J. C., MacLeod, A., & Walters, K. F. A. (2000). The role of climatic mapping in predicting the potential geographical distribution of non-indigenous pests under current and future climates. *Agriculture, Ecosystems and Environment*, *82*(1–3), 57–71. https://doi.org/10.1016/S0167-8809(00)00216-4

Ben, C., Debellé, F., Berges, H., Bellec, A., Jardinaud, M. F., Anson, P., Huguet, T., Gentzbittel, L., & Vailleau, F. (2013). MtQRRS1, an R-locus required for Medicago truncatula quantitative resistance to Ralstonia solanacearum. *New Phytologist*, *199*(3), 758–772. https://doi.org/10.1111/nph.12299

Bender, C. L., Stone, H. E., Sims, J. J., & Cooksey, D. A. (1987). Reduced pathogen fitness of Pseudomonas syringae pv. tomato Tn5 mutants defective in coronatine production. *Physiological and Molecular Plant Pathology*, *30*(2), 273–283. https://doi.org/10.1016/0885-5765(87)90040-3

Bolwell, G. P. (2002). The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *Journal of Experimental Botany*, *53*(372), 1367–1376. https://doi.org/10.1093/jexbot/53.372.1367

Boucher, C. A., Barberis, P. A., Trigalet, A. P., & Demery, D. A. (1985). Transposon mutagenesis of Pseudomonas solanacearum: Isolation of Tn5-induced avirulent mutants. *Journal of General Microbiology*, *131*(9), 2449–2457. https://doi.org/10.1099/00221287-131-9-2449

Boucher, C. A., Van Gijsegem, F., Barberis, P. A., Arlat, M., & Zischek, C. (1987). Pseudomonas solanacearum genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. *Journal of Bacteriology*, *169*(12), 5626–5632. https://doi.org/10.1128/jb.169.12.5626-5632.1987

Boureau, T., Routtu, J., Roine, E., Taira, S., & Romantschuk, M. (2002). Localization of hrpA-induced Pseudomonas syringae pv. tomato DC3000 in infected tomato leaves. *Molecular Plant Pathology*, *3*(6), 451–460. https://doi.org/10.1046/j.1364-3703.2002.00139.x

Brasier, C. M. (2001). Rapid evolution of introduced plant pathogens via interspecific Hybridization. *BioScience*, *51*(2), 123–133. https://doi.org/10.1641/0006-3568(2001)051[0123:REOIPP]2.0.CO;2

Bray, E. A., Bailey-Serres, J., & Weretilnyk, E. (2000). Responses to abiotic stresses. In *Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville* (pp. 1158–1249).

Brito, B., Aldon, D., Barberis, P., Boucher, C., & Genin, S. (2002). A signal transfer system through three compartments transduces the plant cell contact-dependent signal controlling Ralstonia solanacearum hrp genes. *Molecular Plant-Microbe Interactions*, *15*(2), 109–119. https://doi.org/10.1094/MPMI.2002.15.2.109

Brito, B., Marenda, M., Barberis, P., Boucher, C., & Genin, S. (1999). PrhJ and hrpG, two new components of the plant signal-dependent regulatory cascade controlled by PrhA in Ralstonia solanacearum. *Molecular Microbiology*, *31*(1), 237–251. https://doi.org/10.1046/j.1365-2958.1999.01165.x

Brooks, D. M., Bender, C. L., & Kunkel, B. N. (2005). The Pseudomonas syringae phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in Arabidopsis thaliana. *Molecular Plant Pathology*, *6*(6), 629–639. https://doi.org/10.1111/j.1364-3703.2005.00311.x

Brown, D. G., & Allen, C. (2004). Ralstonia solanacearum genes induced during growth in tomato: An inside view of bacterial wilt. *Molecular Microbiology*, *53*(6), 1641–1660. https://doi.org/10.1111/j.1365-2958.2004.04237.x

Brown, D. G., Swanson, J. K., & Allen, C. (2007). Two host-induced Ralstonia solanacearum genes, acrA and dinF, encode multidrug efflux pumps and contribute to bacterial wilt virulence. *Applied and Environmental Microbiology*, *73*(9), 2777–2786. https://doi.org/10.1128/AEM.00984-06

Büttner, D., & Bonas, U. (2010a). Regulation and secretion of Xanthomonas virulence factors. *FEMS Microbiology Reviews*, *34*(2), 107–133. https://doi.org/10.1111/j.1574-6976.2009.00192.x

Büttner, D., & Bonas, U. (2010b). Regulation and secretion of Xanthomonas virulence factors. In *FEMS Microbiology Reviews* (Vol. 34, Issue 2, pp. 107–133). Oxford Academic. https://doi.org/10.1111/j.1574-6976.2009.00192.x

Büttner, D., & He, S. Y. (2009). Type III protein secretion in plant pathogenic bacteria. *Plant Physiology*, *150*(4), 1656–1664. https://doi.org/10.1104/pp.109.139089

Caldwell, D. (2016). *The Role of Root Anatomy and Root Architecture in Resistance to Ralstonia solanacearum* (Issue January).

Caldwell, D., Kim, B. S., & Iyer-Pascuzzi, A. S. (2017). Ralstonia solanacearum differentially colonizes roots of resistant and susceptible tomato plants. *Phytopathology*, *107*(5), 528–536. https://doi.org/10.1094/PHYTO-09-16-0353-R

Caldwell, R. M., Schafer, J. F., Compton, L. E., & Patterson, F. L. (1958). Tolerance to Cereal Leaf Rusts Author (s): Ralph M . Caldwell , John F . Schafer , Leroy E . Compton and Fred L . Patterson Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/1756206 Accessed : 07. *Science*, *128*(3326), 714–715.

Cardinale, M., Puglia, A. M., & Grube, M. (2006). Molecular analysis of lichen-associated bacterial communities. *FEMS Microbiology Ecology*, *57*(3), 484–495. https://doi.org/10.1111/j.1574-6941.2006.00133.x

Carputo, D., Aversano, R., Barone, A., Di Matteo, A., Iorizzo, M., Sigillo, L., Zoina, A., & Frusciante, L. (2009). Resistance to ralstonia solanacearum of sexual hybrids between solanum commersonii and S. tuberosum. *American Journal of Potato Research*, *86*(3), 196–202. https://doi.org/10.1007/s12230-009-9072-4

Caruso, P., Palomo, J. L., Bertolini, E., Álvarez, B., López, M. M., & Biosca, E. G. (2005). Seasonal variation of Ralstonia solanacearum biovar 2 populations in a Spanish river: Recovery of stressed cells at low temperatures. *Applied and Environmental Microbiology*, *71*(1), 140–148. https://doi.org/10.1128/AEM.71.1.140-148.2005

Caruso, P., Palomo, J. L., Bertolini, E., López, M. M., & Biosca, E. G. (2005). *Seasonal Variation of Ralstonia solanacearum Biovar 2 Populations in a Spanish River : Recovery of Stressed Cells at Low Temperatures Seasonal Variation of Ralstonia solanacearum Biovar 2 Populations in a Spanish River : Recovery of Stressed Cells at Low* . *71*(1), 140–148. https://doi.org/10.1128/AEM.71.1.140

Chakrabarti, S. K., Shekhawat, G. S., Gadewar, A. V., & Gopal, J. (1993). Selection of bacterial wilt tolerant potato germplasm. In *Seminar on crop protection*.

Champoiseau, P. G., Jones, J. B., & Allen, C. (2009). Ralstonia solanacearum Race 3 Biovar 2 Causes Tropical Losses and Temperate Anxieties . *Plant Health Progress*, *10*(1), 35.

https://doi.org/10.1094/php-2009-0313-01-rv

Chatterjee, S., Newman, K. L., & Lindow, S. E. (2008). Cell-to-cell signaling in Xylella fastidiosa suppresses movement and xylem vessel colonization in grape. *Molecular Plant-Microbe Interactions*, *21*(10), 1309–1315. https://doi.org/10.1094/MPMI-21-10-1309

Chatterjee, S., Wistrom, C., & Lindow, S. E. (2008). A cell-cell signaling sensor is required for virulence and insect transmission of Xylella fastidiosa. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(7), 2670–2675. https://doi.org/10.1073/pnas.0712236105

Chen, D., Liu, B., Zhu, Y., Jieping, W., Zheng, C., Che, J., Zheng, X., & Chen, X. (2017). Complete genome sequence of Ralstonia solanacearum FJAT-1458, a potential biocontrol agent for tomato wilt. *Genome Announcements*, *5*(14), 4–5.

Child, A., & Lester, R. N. (2001). Synopsis of the genus Solanum L. and its infrageneric taxa. In R. G. Van den Berg, G. W. M. Barendse, G. M. Van der Weerden, & C. Mariani (Eds.), *Solanaceae V: Advances in taxonomy and utilization. Nijmegen University Press* (p. 43).

Ciampi, L., Sequeira, L., & French, E. R. (1980). Latent infection of potato tubers by Pseudomonas solanacearum. *American Potato Journal*, *57*(8), 377–386. https://doi.org/10.1007/BF02854329

Clarke, D. D. (1986). Tolerance of parasites and disease in plants and its significance in host-parasite interactions. *Advances in Plant Pathology*, *5*, 161–197.

Clough, S. J., Schell, M. A., & Denny, T. P. (1994). Evidence for involvement of a volatile extracellular factor in Pseudomonas solanacearum virulence gene expression. In *Molecular Plant-Microbe Interactions* (Vol. 7, Issue 5, pp. 621–630). https://doi.org/10.1094/MPMI-7-0621

Clough, Steven J., Flavier, A. B., Schell, M. A., & Denny, T. P. (1997). Differential expression of virulence genes and motility in Ralstonia (Pseudomonas) solanacearum during exponential growth. *Applied and Environmental Microbiology*, *63*(3), 844–850. https://doi.org/10.1128/aem.63.3.844-850.1997

Clough, Steven J., Lee, K. E., Schell, M. A., & Denny, T. P. (1997). A two-component system in Ralstonia (Pseudomonas) solanacearum modulates production of PhcA-regulated virulence factors in response to 3- hydroxypalmitic acid methyl ester. *Journal of Bacteriology*, *179*(11), 3639–3648. https://doi.org/10.1128/jb.179.11.3639-3648.1997

Coburn, B., Sekirov, I., & Finlay, B. B. (2007). Type III secretion systems and disease. *Clinical Microbiology Reviews*, *20*(4), 535–549. https://doi.org/10.1128/CMR.00013-07

Coll, N. S., & Valls, M. (2013). Current knowledge on the Ralstonia solanacearum type III secretion system. *Microbial Biotechnology*, *6*(6), 614–620. https://doi.org/10.1111/1751-7915.12056

Collonnier, C., Mulya, K., Fock, I., Mariska, I., Servaes, A., Vedel, F., Siljak-Yakovlev, S., Souvannavong, V., Ducreux, G., & Sihachakr, D. (2001). Source of resistance against Ralstonia solanacearum in fertile somatic hybrids of eggplant (Solanum melongena L.) with Solanum aethiopicum L. *Plant Science*, *160*(2), 301–313. https://doi.org/10.1016/S0168-9452(00)00394-0

Constantinidou, H. A., Upper, C. D., Lindemann, J., & Barchet, W. R. (1982). Plants as sources of airborne bacteria, including ice nucleation-active bacteria. *Applied and Environmental Microbiology*, *44*(5), 1059–1063.

Conway, J. R., Lex, A., & Gehlenborg, N. (2017). UpSetR: An R package for the visualization of intersecting sets and their properties. *Bioinformatics*, *33*(18), 2938–2940. https://doi.org/10.1093/bioinformatics/btx364

Corral, J., Sebastià, P., Coll, N. S., Barbé, J., Aranda, J., & Valls, M. (2020). Twitching and Swimming Motility Play a Role in Ralstonia solanacearum Pathogenicity . *MSphere*, *5*(2), 1–16. https://doi.org/10.1128/msphere.00740-19

Cruz, A. P. Z., Ferreira, V., Pianzzola, M. J., Siri, M. I., Coll, N. S., & Valls, M. (2014). A novel, sensitive method to evaluate potato germplasm for bacterial wilt resistance using a luminescent ralstonia solanacearum reporter strain. *Molecular Plant-Microbe Interactions*, *27*(3), 277–285. https://doi.org/10.1094/MPMI-10-13-0303-FI

Cunnac, S., Occhialini, A., Barberis, P., Boucher, C., & Genin, S. (2004). Inventory and functional analysis of the large Hrp regulon in Ralstonia solanacearum: Identification of novel effector proteins translocated to plant host cells through the type III secretion system. *Molecular Microbiology*, *53*(1), 115–128. https://doi.org/10.1111/j.1365-2958.2004.04118.x

Dalsing, B. L., & Allen, C. (2014). Nitrate assimilation contributes to Ralstonia solanacearum root attachment, stem colonization, and virulence. *Journal of Bacteriology*, *196*(5), 949–960. https://doi.org/10.1128/JB.01378-13

Dalsing, B. L., Truchon, A. N., Gonzalez-Orta, E. T., Milling, A. S., & Allen, C. (2015). Ralstonia solanacearum uses inorganic nitrogen metabolism for virulence, ATP production, and detoxification in the oxygen-limited host xylem environment. *MBio*, *6*(2), 1–13. https://doi.org/10.1128/mBio.02471-14

Danhorn, T., & Fuqua, C. (2007). Biofilm formation by plant-associated bacteria. *Ann. Rev. Phytopathol*, *61*, 401–422.

Daugherty, M. P., Rashed, A., Almeida, R. P. P., & Perring, T. M. (2011). Vector preference for hosts differing in infection status: Sharpshooter movement and Xylella fastidiosa transmission. *Ecological Entomology*, *36*(5), 654–662. https://doi.org/10.1111/j.1365-2311.2011.01309.x

de Bary, A. (1876). Researches into the nature of the potato fungus Phytophthora infestans. *Journal of the Royal Agricultural Society of England*, *12*, 239–269.

Denance, N., Legendre, B., Briand, M., Olivier, V., de Boisseson, C., Poliakoff, F., & Jacques, M.-A. (2017). Several subspecies and sequence types are associated with the emergene of Xylella fastidiosa in natural settings in France. *Plant Pathology Journal*, *66*, 1054–1064. https://doi.org/10.1111/ijlh.12426

Denny, T. (2006). Plant pathogenic Ralstonia species. In *Plant-associated bacteria* (pp. 573–644). Springer Netherlands.

Denny, T. P. and S.-R. B. (1991). *Genetic evidence that extracellular polysaccharide is a virulence factor of Pseudomonas solanacearum* (Vol. 4, Issue 2, pp. 198–206). https://doi.org/10.1094/MPMI-4-198

Denny, T. P., Carney, B. F., & Schell, M. A. (1990). Inactivation of multiple virulence genes reduces the ability of Pseudomonas solanacearum to cause wilt symptoms. *Molecular Plant-Microbe Interactions*, *3*, 293–300.

Deslandes, L., Olivier, J., Peeters, N., Feng, D. X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S., & Marco, Y. (2003). Physical interaction between RRS1-R, a protein conferring

resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(13), 8024–8029. https://doi.org/10.1073/pnas.1230660100

Desseaux, Y. (1993). Chemistry and biochemistry of opines, chemical mediators of parasitism. *Phytochemistry*, *34*, 31–38.

Devash, Y., Okon, Y., & Henis, Y. (1980). Survival of Pseudomonas tomato in soil and seeds. *Phytopathology*, *99*, 175–185.

Digonnet, C., Martinez, Y., Denancé, N., Chasseray, M., Dabos, P., Ranocha, P., Marco, Y., Jauneau, A., & Goffner, D. (2012). Deciphering the route of Ralstonia solanacearum colonization in Arabidopsis thaliana roots during a compatible interaction: Focus at the plant cell wall. *Planta*, *236*(5), 1419–1431. https://doi.org/10.1007/s00425-012-1694-y

Du, Y., Stegmann, M., & Misas-Villamil, J. C. (2015). *Meetings The apoplast as battleground for plant – microbe interactions*.

Elizabeth, S. V., & Bender, C. L. (2007). The phytotoxin coronatine from Pseudomonas syringae pv. tomato DC3000 functions as a virulence factor and influences defence pathways in edible brassicas. *Molecular Plant Pathology*, *8*(1), 83–92. https://doi.org/10.1111/j.1364-3703.2006.00372.x

Elphinstone, J. G. (1996). Survival and possibilities for extinction of Pseudomonas solanacearum (Smith) Smith in cool climates. *Potato Research*, *39*(SPEC. ISS.), 403–410. https://doi.org/10.1007/bf02357946

Elphinstone, J. G., Stanford, H., Stead, D. E., & Hutton, S. (1998). Survival and transmission of Ralstonia solanacearum in aquatic plants of Solanum dulcamara and associated surface water in England. *EPPO Bulletin*, *94*(28), 93–94.

Esau, K. (1977). *Anatomy of Seed Plants, 2nd Edition*.

Escobar, M. A., & Dandekar, A. M. (2003). Agrobacterium tumefaciens as an agent of disease. *Trends in Plant Science*, *8*(8), 380–386. https://doi.org/10.1016/S1360-1385(03)00162-6

Felix, R., Onyango, O. J., & Eliazer, O. M. (2010). Assesment of Irish potato cultivars field tolerance to bacterial wilt (Ralstonia solanacearum) in Kenya. *Plant Pathology Journal*, *9*(3), 122–128.

Flavier, A. B., Clough, S. J., Schell, M. A., & Denny, T. P. (1997). Identification of 3-hydroxypalmitic acid methyl ester as a novel autoregulator controlling virulence in Ralstonia solanacearum. *Molecular Microbiology*, *26*(2), 251–259. https://doi.org/10.1046/j.1365-2958.1997.5661945.x

Flavier, A. B., Ganova-Raeva, L. M., Schell, M. A., & Denny, T. P. (1997). Hierarchical autoinduction in Ralstonia solanacearum: Control of acyl- homoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester. *Journal of Bacteriology*, *179*(22), 7089–7097. https://doi.org/10.1128/jb.179.22.7089-7097.1997

Flavier, A. B., Schell, M. A., & Denny, T. P. (1998). An RpoS (σ(s)) homologue regulates acylhomoserine lactone-dependent autoinduction in Ralstonia solanacearum. *Molecular Microbiology*, *28*(3), 475–486. https://doi.org/10.1046/j.1365-2958.1998.00804.x

Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. In *Nature Reviews Microbiology* (Vol. 8, Issue 9, pp. 623–633). Nature Publishing Group.

https://doi.org/10.1038/nrmicro2415

Flores-Cruz, Z., & Allen, C. (2009). Ralstonia solanacearum encounters an oxidative environment during tomato infection. *Molecular Plant-Microbe Interactions*, *22*(7), 773–782. https://doi.org/10.1094/MPMI-22-7-0773

Flores-Cruz, Z., & Allen, C. (2011). Necessity of OxyR for the hydrogen peroxide stress response and full virulence in Ralstonia solanacearum. *Applied and Environmental Microbiology*, *77*(18), 6426–6432. https://doi.org/10.1128/AEM.05813-11

Francl, L. J. (2001). *The Disease Triangle: a plant pathological paradigm revisited*. The Plant Health Instructor.

Frank, S. A. (1996). Models of Parasite Virulence. *The University of Chicago Press Journals*, *71*(1), 37–78.

Frantzen, J. (2007). *Epidemiology and Plant Ecology: Principles and Applications*. Singapore: World Sci.

Freebairn, H. T., & Buddenhagen, I. W. (1964). Ethylene production by Pseudomonas solanacearum. *Nature*, 313.314.

Fujiwara, S., Kawazoe, T., Ohnishi, K., Kitagawa, T., Popa, C., Valls, M., Genin, S., Nakamura, K., Kuramitsu, Y., Tanaka, N., & Tabuchi, M. (2016). RipAY, a Plant Pathogen Effector Protein, Exhibits Robust γ-Glutamyl Cyclotransferase Activity When Stimulated by Eukaryotic Thioredoxins. *Journal of Biological Chemistry*, *291*(13), 6813–6830. https://doi.org/10.1074/jbc.M115.678953

Garg, R. P., Huang, J., Yindeeyoungyeon, W., Denny, T. P., & Schell, M. A. (2000). Multicomponent transcriptional regulation at the complex promoter of the exopolysaccharide I biosynthetic operon of Ralstonia solanacearum. *Journal of Bacteriology*, *182*(23), 6659–6666. https://doi.org/10.1128/JB.182.23.6659-6666.2000

Garrett, K. A., Dendy, S. P., Frank, E. E., Rouse, M. N., & Travers, S. E. (2006). Climate change effects on plant disease: Genomes to ecosystems. *Annual Review of Phytopathology*, *44*(February), 489–509. https://doi.org/10.1146/annurev.phyto.44.070505.143420

Gelvin, S. B. (2000). *AGROBACTERIUM AND PLANT GENES INVOLVED IN T-DNA TRANSFER AND INTEGRATION*.

Genin, S. (2010). Molecular traits controlling host range and adaptation to plants in Ralstonia solanacearum. *New Phytologist*, *187*(4), 920–928. https://doi.org/10.1111/j.1469-8137.2010.03397.x

Genin, S., & Boucher, C. (2002). Ralstonia solanacearum: Secrets of a major pathogen unveiled by analysis of its genome. *Molecular Plant Pathology*, *3*(3), 111–118. https://doi.org/10.1046/j.1364-3703.2002.00102.x

Genin, S., Brito, B., Denny, T. P., & Boucher, C. (2005). Control of the Ralstonia solanacearum Type III secretion system (Hrp) genes by the global virulence regulator PhcA. *FEBS Letters*, *579*(10), 2077–2081. https://doi.org/10.1016/j.febslet.2005.02.058

Genin, S., & Denny, T. P. (2012). Pathogenomics of the ralstonia solanacearum species complex. *Annual Review of Phytopathology*, *50*, 67–89. https://doi.org/10.1146/annurev-phyto-081211-173000

Golas, T. M. (2010). *European Solanum dulcamara L . and its interaction with Phytophthora infestans (Mont .) de Bary*.

Golas, T. M., Feron, R. M. C., van den Berg, R. G., van der Weerden, G. M., Mariani, C., & Allefs, J. J. H. M. (2010). Genetic structure of European accessions of Solanum dulcamara L. (Solanaceae). *Plant Systematics and Evolution*, *285*(1), 103–110. https://doi.org/10.1007/s00606-009-0260-y

Golas, Tomasz Michal, Sikkema, A., Gros, J., Feron, R. M. C., van den Berg, R. G., van der Weerden, G. M., Mariani, C., & Allefs, J. J. H. M. (2010). Identification of a resistance gene Rpi-dlc1 to Phytophthora infestans in European accessions of Solanum dulcamara. *Theoretical and Applied Genetics*, *120*(4), 797–808. https://doi.org/10.1007/s00122-009-1202-3

Golas, Tomek M., van der Weerden, G. M., van den Berg, R. G., Mariani, C., & Allefs, J. J. H. M. (2010). Role of Solanum dulcamara L. in Potato Late Blight Epidemiology. *Potato Research*, *53*(1), 69–81. https://doi.org/10.1007/s11540-010-9151-4

González, C. F. (1991). Leersia hexandra, an alternative host for Xanthomonas campestris pv. oryzae in Texas. *The American Phytopathological Society*, *75*(2), 159–162.

González, E. T., & Allen, C. (2003). Characterization of a Ralstonia solanacearum operon required for polygalacturonate degradation and uptake of galacturonic acid. *Molecular Plant-Microbe Interactions*, *16*(6), 536–544. https://doi.org/10.1094/MPMI.2003.16.6.536

González, E. T., Brown, D. G., Swanson, J. K., & Allen, C. (2007). Using the Ralstonia solanacearum Tat secretome to identify bacterial wilt virulence factors. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, *73*(12), 3779–3786. https://doi.org/10.1128/AEM.02999-06

González, M., Galván, G., Siri, M. I., Borges, A., & Vilaró, F. (2013). Resistencia a la marchitez bacteriana de la papa en Solanum commersonii Dun. *Agrociencia Uruguay*, *17*(1), 45–54. https://doi.org/10.2477/vol17iss1pp45-54

Goode, M. J., & Sasser, M. (1980). Prevention-The key to controlling bacterial spot and bacterial speck of tomato. *Plant Disease*, *64*, 831–834.

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., Robles, M., Talón, M., Dopazo, J., & Conesa, A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research*, *36*(10), 3420–3435. https://doi.org/10.1093/nar/gkn176

Gough, C. L., Genin, S., Zischek, C., & Boucher, C. A. (1992). hrp genes of Pseudomonas solanacearum are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. *Molecular Plant-Microbe Interactions : MPMI*, *5*(5), 384–389. https://doi.org/10.1094/MPMI-5-384

Graham, J., Jones, D. A., & Lloyd, A. B. (1979). Survival of Pseudomonas solanacearum Race 3 in Plant Debris and in Latently Infected Potato Tubers. In *Phytopathology* (Vol. 69, Issue 10, p. 1100). https://doi.org/10.1094/phyto-69-1100

Graham, J., & Lloyd, A. B. (1978). Solanum cinereum R. Br., a wild host of Pseudomonas solanacearum biotype II. *Journal of the Australian Institute of Agricultural Science*, *44*(2), 124–126.

Grey, B. G., & Steck, T. R. (2001). The Viable But Nonculturable State of Ralstonia solanacearum may be involved in long-term survival and plant infection. *Applied and Environmental Microbiology*, *67*(9), 3866–3872. https://doi.org/10.1128/AEM.67.9.3866

Grimault, V., & Prior, P. (1993). Bacterial wilt resistance in tomato associated with tolerance of vascular tissues to Pseudomonas solanacearum. *Plant Pathology*, *42*(4), 589–594. https://doi.org/10.1111/j.1365-3059.1993.tb01539.x

Guarischi-Sousa, R., Puigvert, M., Coll, N. S., Siri, M. I., Pianzzola, M. J., Valls, M., & Setubal, J. C. (2016). Complete genome sequence of the potato pathogen Ralstonia solanacearum UY031. *Standards in Genomic Sciences*, *11*(1), 1–8. https://doi.org/10.1186/s40793-016-0131-4

Gunn, C. R., & Gaffney, F. B. (1974). *Seed characteristics of 42 economically important species of Solanaceae in the United States* (T. Bull. (ed.)). Washington, DC: USDA.

Guo, Y. L., & Ge, S. (2005). Molecular phylogeny of Oryzae (Poaceae) based on DNA sequences from chloroplast, mitochondrial, and nuclear genomes. *American Journal of Botany*, *92*(9), 1548–1558. https://doi.org/10.3732/ajb.92.9.1548

Haydon, D. T., Cleaveland, S., Taylor, L. H., & Laurenson, M. K. (2002). Identifying reservoirs of infection: A conceptual and practical challenge. *Emerging Infectious Diseases*, *8*(12), 1468–1473. https://doi.org/10.3201/eid0812.010317

Hayes, M. M., MacIntyre, A. M., & Allen, C. (2017). Complete Genome Sequences of the Plant Pathogens Ralstonia solanacearum Type Strain K60 and R. solanacearum Race 3 Biovar 2 Strain UW551. *Genome Announcements*, *5*(40), 1–2.

Hayward, A. C. (1975). Biotypes of Pseudomonas solanacearum in Australia. *Australasian Plant Pathology*, *4*(2), 9–11.

Hayward, A. C. (1991). Bacterial Wilt Caused by pseudomonas solanacearum. *Annu Rev Phytopathol*, *29*, 65–87. https://doi.org/10.1146/annurev.py.29.090191.000433

Hernández-Romero, D., Solano, F., & Sanchez-Amat, A. (2005). Polyphenol oxidase activity expression in Ralstonia solanacearum. *Applied and Environmental Microbiology*, *71*(11), 6808–6815. https://doi.org/10.1128/AEM.71.11.6808-6815.2005

Hikichi, Y. (2016). Interactions between plant pathogenic bacteria and host plants during the establishment of susceptibility. In *Journal of General Plant Pathology* (Vol. 82, Issue 6, pp. 326–331). Springer Tokyo. https://doi.org/10.1007/s10327-016-0680-9

Hikichi, Y., Yoshimochi, T., Tsujimoto, S., Shinohara, R., Nakaho, K., Kanda, A., Kiba, A., & Ohnishi, K. (2007). Global regulation of pathogenicity mechanism of Ralstonia solanacearum. *Plant Biotechnology*, *24*(1), 149–154. https://doi.org/10.5511/plantbiotechnology.24.149

Hirsch, J., Deslandes, L., Feng, D. X., Balagué, C., & Marco, Y. (2002). Delayed symptom development in ein2-1, an Arabidopsis ethylene-insensitive mutant, in response to bacterial wilt caused by Ralstonia solanacearum. *Phytopathology*, *92*(10), 1142–1148. https://doi.org/10.1094/PHYTO.2002.92.10.1142

Hong, J. C., Momol, M. T., Jones, J. B., Ji, P., Olson, S. M., Allen, C., Perez, A., Pradhanang, P., & Guven, K. (2008). Detection of Ralstonia solanacearum in irrigation ponds and aquatic weeds associated with the ponds in North Florida. *Plant Disease*, *92*(12), 1674–1682. https://doi.org/10.1094/PDIS-92-12-1674

Hong, J. K., Kim, H. J., Jung, H., Yang, H. J., Kim, D. H., Sung, C. H., Park, C. J., & Chang, S. W. (2016). Differential control efficacies of vitamin treatments against bacterial wilt and grey mould diseases in tomato plants. *Plant Pathology Journal*, *32*(5), 469–480. https://doi.org/10.5423/PPJ.OA.03.2016.0076

Hopkins, D. L. (1989). Xylella fastidiosa: xylem-limited bacterial pathogen of plants. *Annual Review of Phytopathology. Vol. 27*, 271–290. https://doi.org/10.1146/annurev.py.27.090189.001415

Horvath, I., Bernath, J., & Tetenyi, P. (1977). Effect of the spectral composition of light on dry matter production in Solanum dulcamara ecotypes of different origin. *Acta Agron Acad Sci*, *26*, 346–354.

Huang, J., Carney, B. F., Denny, T. P., Weissinger, A. K., & Schell, M. A. (1995). A complex network regulates expression of eps and other virulence genes of Pseudomonas solanacearum. *Journal of Bacteriology*, *177*(5), 1259–1267. https://doi.org/10.1128/jb.177.5.1259-1267.1995

Huang, J., Denny, T. P., & Schell, M. A. (1993). VsrB, a regulator of virulence genes of Pseudomonas solanacearum, is homologous to sensors of the two-component regulator family. *Journal of Bacteriology*, *175*(19), 6169–6178. https://doi.org/10.1128/jb.175.19.6169-6178.1993

Huang, Jianzhong, & Schell, M. (1995). Molecular characterization of the eps gene cluster of Pseudomonas solanacearum and its transcriptional regulation at a single promoter. *Molecular Microbiology*, *16*(5), 977–989. https://doi.org/10.1111/j.1365-2958.1995.tb02323.x

Huang, Q., & Allen, C. (1997). An exo-poly-α-D-galacturonosidase, PehB, is required for wild-type virulence of Ralstonia solanacearum. *Journal of Bacteriology*, *179*(23), 7369–7378. https://doi.org/10.1128/jb.179.23.7369-7378.1997

Huang, Qi, & Allen, C. (2000). Polygalacturonases are required for rapid colonization and full virulence of Ralstonia solanacearum on tomato plants. *Physiological and Molecular Plant Pathology*, *57*(2), 77–83. https://doi.org/10.1006/pmpp.2000.0283

Ingram, K. T., & McCloud, D. E. (1984). Simulation of Potato Crop Growth and Development 1 . *Crop Science*, *24*(1), 21–27. https://doi.org/10.2135/cropsci1984.0011183x002400010006x

Ishihara, T., Mitsuhara, I., Takahashi, H., & Nakaho, K. (2012). Transcriptome Analysis of Quantitative Resistance-Specific Response upon Ralstonia solanacearum Infection in Tomato. *PLoS ONE*, *7*(10). https://doi.org/10.1371/journal.pone.0046763

Jacobs, J. M., Babujee, L., Meng, F., Physiological, C., Strategies, V., Wilt, B., This, S., Feeds, R. S. S., & Journal, A. S. M. (2012). *The In Planta Transcriptome of Ralstonia solanacearum : Conserved*. *3*(4), 1–11. https://doi.org/10.1128/mBio.00114-12.Editor

Jacobs, J. M., Milling, A., Mitra, R. M., Hogan, C. S., Ailloud, F., Prior, P., & Allen, C. (2013). Ralstonia solanacearum requires pops, an ancient avre-family effector, for virulence and to overcome salicylic acid-mediated defenses during tomato pathogenesis. *MBio*, *4*(6). https://doi.org/10.1128/mBio.00875-13

Jang, I. A., Kim, J., & Park, W. (2016). Endogenous hydrogen peroxide increases biofilm formation by inducing exopolysaccharide production in Acinetobacter oleivorans DR1. *Scientific Reports*, *6*(February), 1–12. https://doi.org/10.1038/srep21121

Janse, J. D. (1996). Potato brown rot in western Europe – history, present occurrence and some remarks on possible origin, epidemiology and control strategies. *EPPO Bulletin*, *26*(3–4), 679–695. https://doi.org/10.1111/j.1365-2338.1996.tb01512.x

Janse, J. D., Araluppan, F. A. X., Schans, J., Wenneker, M., & Westerhuis, W. (1998).

Experiences with Bacterial Brown Rot Ralstonia solanacearum Biovar 2, Race 3 in the Netherlands. *Bacterial Wilt Disease*, *September 1995*, 146–152. https://doi.org/10.1007/978-3-662-03592-4_21

Janse, J. D., Van Den Beld, H. E., Elphinstone, J., Simpkins, S., Tjou-Tam-Sin, N. N. A., & Van Vaerenbergh, J. (2004). Introduction to Europe of Ralstonia solanacearum biovar 2, race 3 in Pelargonium zonale cuttings. *Journal of Plant Pathology*, *86*(2), 147–155.

Jeon, H., Kim, W., Kim, B., Lee, S., Jayaraman, J., Jung, G., Choi, S., Sohn, K. H., & Segonzac, C. (2020). Erratum: Ralstonia solanacearum Type III effectors with predicted nuclear localization signal localize to various cell compartments and modulate immune responses in Nicotiana spp. (Plant Pathol. J., (2020) 36(1), (43-53), 10.5423/PPJ.OA.08.2019.0227). *Plant Pathology Journal*, *36*(3), 303. https://doi.org/10.5423/PPJ.ER.08.2019.0227

Kai, K., Ohnishi, H., Mori, Y., Kiba, A., Ohnishi, K., & Hikichi, Y. (2014). Involvement of ralfuranone production in the virulence of ralstonia solanacearum OE1-1. *ChemBioChem*, *15*(17), 2590–2597. https://doi.org/10.1002/cbic.201402404

Kanda, A., Ohnishi, K., Kiba, A., & Hikichi, Y. (2009). Implication of C-terminal mutation of PopA of Ralstonia solanacearum strain OE1-1 in suppression of bacterial wilt. *Plant Pathology*, *58*(1), 159–169. https://doi.org/10.1111/j.1365-3059.2008.01938.x

Kanda, Ayami, Ohnishi, S., Tomiyama, H., Hasegawa, H., Yasukohchi, M., Kiba, A., Ohnishi, K., Okuno, T., & Hikichi, Y. (2003). Type III secretion machinery-deficient mutants of Ralstonia solanacearum lose their ability to colonize resulting in loss of pathogenicity. *Journal of General Plant Pathology*, *69*(4), 250–257. https://doi.org/10.1007/s10327-003-0041-3

Kanda, Ayami, Tsuneishi, K., Mori, A., Ohnishi, K., Kiba, A., & Hikichi, Y. (2008). An amino acid substitution at position 740 in σ70 of Ralstonia solanacearum strain OE1-1 affects its in planta growth. *Applied and Environmental Microbiology*, *74*(18), 5841–5844. https://doi.org/10.1128/AEM.01099-08

Kanda, Ayami, Yasukohchi, M., Ohnishi, K., Kiba, A., Okuno, T., & Hikichi, Y. (2003). Ectopic expression of Ralstonia solanacearum effector protein popA early in invasion results in loss of virulence. *Molecular Plant-Microbe Interactions*, *16*(5), 447–455. https://doi.org/10.1094/MPMI.2003.16.5.447

Kang, Y., Liu, H., Genin, S., Schell, M. A., & Denny, T. P. (2002a). Ralstonia solanacearum requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. *Molecular Microbiology*, *46*(2), 427–437. https://doi.org/10.1046/j.1365-2958.2002.03187.x

Kang, Y., Liu, H., Genin, S., Schell, M. A., & Denny, T. P. (2002b). *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. *Molecular Microbiology*, *46*(2), 427–437. https://doi.org/10.1046/j.1365-2958.2002.03187.x

Kang Yaowei, Huang Jianzhong, Mao Guozhang, He Li yuan, & Schell, M. A. (1994). Dramatically reduced virulence of mutants of Pseudomonas solanacearum defective in export of extracellular proteins across the outer membrane. *Molecular Plant-Microbe Interactions*, *7*(3), 370–377. https://doi.org/10.1094/mpmi-7-0370

Kao, C. C., Barlow, E., & Sequeira, L. (1992). Extracellular polysaccharide is required for wild-type virulence of Pseudomonas solanacearum. *Journal of Bacteriology*, *174*(3), 1068–1071. https://doi.org/10.1128/jb.174.3.1068-1071.1992

Kelman, A. (1954). The relationship of pathogenicity of Pseudomonas solanacearum to colony appearance in tetrazolium medium. *Phytopathology*, *44*(12).

Khokhani, D., Lowe-Power, T. M., Tran, T. M., & Allen, C. (2017). A Single Regulator Mediates Strategic Switching between Attachment / Spread and Growth / Virulence in the Plant. *MBio*, *8*(5), 1–20.

Kudela, V. (2009). Potential impact of climate change on geographic distribution of plant pathogenic bacteria in Central Europe. *Plant Protection Science*, *45*(SPEC. ISS). https://doi.org/10.17221/2832-pps

Kumar, L., & Futschik, M. E. (2007). Mfuzz: A software package for soft clustering of microarray data. *Bioinformation*. https://doi.org/10.6026/97320630002005

Lang, J. M., Pérez-Quintero, A. L., Koebnik, R., DuCharme, E., Sarra, S., Doucoure, H., Keita, I., Ziegle, J., Jacobs, J. M., Oliva, R., Koita, O., Szurek, B., Verdier, V., & Leach, J. E. (2019). A pathovar of Xanthomonas oryzae infecting wild grasses provides insight into the evolution of pathogenicity in rice agroecosystems. *Frontiers in Plant Science*, *10*(April), 1–15. https://doi.org/10.3389/fpls.2019.00507

Langmead, B., & Salzberg, S. (2013). Bowtie2. *Nature Methods*, *9*(4), 357–359. https://doi.org/10.1038/nmeth.1923.Fast

Langner, T., Białas, A., & Kamoun, S. (2018). The blast fungus decoded: Genomes in flux. *MBio*, *9*(2), 1–4. https://doi.org/10.1128/mBio.00571-18

Lebeau, A., Gouy, M., Daunay, M. C., Wicker, E., Chiroleu, F., Prior, P., Frary, A., & Dintinger, J. (2013). Genetic mapping of a major dominant gene for resistance to Ralstonia solanacearum in eggplant. *Theoretical and Applied Genetics*, *126*(1), 143–158. https://doi.org/10.1007/s00122-012-1969-5

Lei, N., Chen, L., Kiba, A., Hikichi, Y., Zhang, Y., Ohnishi, K., & Nishimura, M. T. (2020). *Super-Multiple Deletion Analysis of Type III Effectors in Ralstonia solanacearum OE1-1 for Full Virulence Toward Host Plants Bacterial Strains and Culture Conditions*. *11*(July), 1–10. https://doi.org/10.3389/fmicb.2020.01683

Li, Z., Wu, S., Bai, X., Liu, Y., Lu, J., Liu, Y., Xiao, B., Lu, X., & Fan, L. (2011). Genome sequence of the tobacco bacterial wilt pathogen ralstonia solanacearum. *Journal of Bacteriology*, *193*(21), 6088–6089. https://doi.org/10.1128/JB.06009-11

Liba, C. M., Ferrara, F. I. S., Manfio, G. P., Fantinatti-Garboggini, F., Albuquerque, R. C., Pavan, C., Ramos, P. L., Moreira-Filho, C. A., & Barbosa, H. R. (2006). Nitrogen-fixing chemo-organotrophic bacteria isolated from cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones. *Journal of Applied Microbiology*, *101*(5), 1076–1086. https://doi.org/10.1111/j.1365-2672.2006.03010.x

Lindgren, P. B., Peet, R. C., & Panopoulos, N. J. (1986). Gene cluster of Pseudomonas syringae pv. “phaseolicola” controls pathogenicity of bean plants and hypersensitivity on nonhost plants. *Journal of Bacteriology*, *168*(2), 512–522. https://doi.org/10.1128/jb.168.2.512-522.1986

Lindow, S. E., & Brandl, M. T. (2003). MINIREVIEW Microbiology of the Phyllosphere. *Society*, *69*(4), 1875–1883. https://doi.org/10.1128/AEM.69.4.1875

Liu, H., Kang, Y., Genin, S., Schell, M. A., & Denny, T. P. (2001). Twitching motility of Ralstonia solanacearum requires a type IV pilus system. *Microbiology*, *147*(12), 3215–3229. https://doi.org/10.1099/00221287-147-12-3215

Liu, Huanli, Zhang, S., Schell, M. A., & Denny, T. P. (2005). Pyramiding unmarked deletions in Ralstonia solanacearum shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. *Molecular Plant-Microbe Interactions*, *18*(12), 1296–1305. https://doi.org/10.1094/MPMI-18-1296

Lohou, D., Turner, M., Lonjon, F., Cazalé, A. C., Peeters, N., Genin, S., & Vailleau, F. (2014). HpaP modulates type III effector secretion in Ralstonia solanacearum and harbours a substrate specificity switch domain essential for virulence. *Molecular Plant Pathology*, *15*(6), 601–614. https://doi.org/10.1111/mpp.12119

Lonjon, F., Turner, M., Henry, C., Rengel, D., Lohou, D., Van De Kerkhove, Q., Cazale, A. C., Peeters, N., Genin, S., & Vailleau, F. (2016). Comparative secretome analysis of ralstonia solanacearum type 3 secretion-Associated mutants reveals a fine control of effector delivery, essential for bacterial pathogenicity. *Molecular and Cellular Proteomics*, *15*(2), 598–613. https://doi.org/10.1074/mcp.M115.051078

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 1–21. https://doi.org/10.1186/s13059-014-0550-8

Lovelace, A. H., Smith, A., & Kvitko, B. H. (2018). Pattern-triggered immunity alters the transcriptional regulation of virulence-associated genes and induces the sulfur starvation response in pseudomonas syringae pv. tomato DC3000. *Molecular Plant-Microbe Interactions*, *31*(7), 750–765. https://doi.org/10.1094/MPMI-01-18-0008-R

Lowe-Power, T. M., Hendrich, C. G., von Roepenack-Lahaye, E., Li, B., Wu, D., Mitra, R., Dalsing, B. L., Ricca, P., Naidoo, J., Cook, D., Jancewicz, A., Masson, P., Thomma, B., Lahaye, T., Michael, A. J., & Allen, C. (2018). Metabolomics of tomato xylem sap during bacterial wilt reveals Ralstonia solanacearum produces abundant putrescine, a metabolite that accelerates wilt disease. *Environmental Microbiology*, *20*(4), 1330–1349. https://doi.org/10.1111/1462-2920.14020

Lowe-Power, T. M., Khokhani, D., & Allen, C. (2018). How Ralstonia solanacearum Exploits and Thrives in the Flowing Plant Xylem Environment. *Trends in Microbiology*, *26*(11), 929–942. https://doi.org/10.1016/j.tim.2018.06.002

Lu, H., Lema, S. A., Planas-Marquès, M., Alonso-Díaz, A., Valls, M., & Coll, N. S. (2018). Type III secretion-dependent and-independent phenotypes caused by Ralstonia solanacearum in Arabidopsis roots. *Molecular Plant-Microbe Interactions*, *31*(1), 175–184. https://doi.org/10.1094/MPMI-05-17-0109-FI

Macho, A. P., & Zipfel, C. (2015). Targeting of plant pattern recognition receptor-triggered immunity by bacterial type-III secretion system effectors. *Current Opinion in Microbiology*, *23*, 14–22.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S. V., Machado, M. A., Toth, I., Salmond, G., & Foster, G. D. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology*, *13*(6), 614–629. https://doi.org/10.1111/j.1364-3703.2012.00804.x

Marenda, M., Brito, B., Callard, D., Genin, S., Barberis, P., Boucher, C., & Arlat, M. (1998). PrhA controls a novel regulatory pathway required for the specific induction of Ralstonia solanacearum hrp genes in the presence of plant cells. *Molecular Microbiology*, *27*(2), 437–453. https://doi.org/10.1046/j.1365-2958.1998.00692.x

Martins, P. M. M., Merfa, M. V., Takita, M. A., & De Souza, A. A. (2018). Persistence in

phytopathogenic bacteria: Do we know enough? *Frontiers in Microbiology*, *9*(MAY), 1–14. https://doi.org/10.3389/fmicb.2018.01099

Masini, L., Grenville-Briggs, L. J., Andreasson, E., Råberg, L., & Lankinen, Å. (2019). Tolerance and overcompensation to infection by Phytophthora infestans in the wild perennial climber Solanum dulcamara. *Ecology and Evolution*, *9*(8), 4557–4567. https://doi.org/10.1002/ece3.5057

Mattick, J. S. (2002). Type IV pili and twitching motility. *Annual Review of Microbiology*, *56*, 289–314. https://doi.org/10.1146/annurev.micro.56.012302.160938

McCarter, S. M. (1983). Survival of Pseudomonas syringae pv. tomato in Association with Tomato Seed, Soil, Host Tissue, and Epiphytic Weed Hosts in Georgia . *Phytopathology*, *73*(10), 1393. https://doi.org/10.1094/phyto-73-1393

McGarvey, J., Bell, C., Denny, T., & Schell, M. (1998). Analysis of extracellular polysaccharide I in culture and in planta using immunological methods: new insights and implications. In *Bacterial Wilt Disease* (pp. 157–163). Springer.

McNew, G. L. (1960). The nature, origin, and evolution of parasitism. In *Plant Pathology: An Advanced Treatise* (pp. 19–69).

Melotto, M., Underwood, W., Koczan, J., Nomura, K., & He, S. Y. (2006). Plant Stomata Function in Innate Immunity against Bacterial Invasion. *Cell*, *126*(5), 969–980. https://doi.org/10.1016/j.cell.2006.06.054

Meng, F., Babujee, L., Jacobs, J. M., & Allen, C. (2015). Comparative transcriptome analysis reveals cool virulence factors of Ralstonia solanacearum race 3 biovar 2. *PLoS ONE*, *10*(10), 1–22. https://doi.org/10.1371/journal.pone.0139090

Meng, F., Yao, J., & Allen, C. (2011). A MotN mutant of Ralstonia solanacearum is hypermotile and has reduced virulence. *Journal of Bacteriology*, *193*(10), 2477–2486. https://doi.org/10.1128/JB.01360-10

Mew, T. W. (1993). Focus on Bacterial Blight of Rice. *Plant Disease*, *77*(1), 5. https://doi.org/10.1094/pd-77-0005

Meyer, D., Cunnac, S., Guéneron, M., Declercq, C., Van Gijsegem, F., Lauber, E., Boucher, C., & Arlat, M. (2006). PopF1 and PopF2, two proteins secreted by the Type III protein secretion system of Ralstonia solanacearum, are translocators belonging to the HrpF/NopX family. *Journal of Bacteriology*, *188*(13), 4903–4917. https://doi.org/10.1128/JB.00180-06

Milling, A., Meng, F., Denny, T. P., & Allen, C. (2009). Interactions with hosts at cool temperatures, not cold tolerance, explain the unique epidemiology of ralstonia solanacearum race 3 biovar 2. *Phytopathology*, *99*(10), 1127–1134. https://doi.org/10.1094/PHYTO-99-10-1127

Mizukami, T., & Wakimoto, S. (1969). Epidemiology and control of bacterial leaf blight of rice. *Annual Review of Phytopathology*, *7*, 51–72.

Moffett, M. L., & Hayward, A. C. (1980). The role of weed species in the survival of Pseudomonas solanacearum in tomato cropping land. *Australasian Plant Pathology*, *9*(2), 6–8. https://doi.org/10.1071/APP98020006

Montanaro, L., Poggi, A., Visai, L., Ravaioli, S., Campoccia, D., Speziale, P., & Arciola, C. R. (2011). Extracellular DNA in biofilms. *International Journal of Artificial Organs*, *34*(9),

824–831. https://doi.org/10.5301/ijao.5000051

Monteil, C. L., Cai, R., Liu, H., Mechan Llontop, M. E., Leman, S., Studholme, D. J., Morris, C. E., & Vinatzer, B. A. (2013). Nonagricultural reservoirs contribute to emergence and evolution of Pseudomonas syringae crop pathogens. *New Phytologist*, *199*(3), 800–811. https://doi.org/10.1111/nph.12316

Monteiro, F., Genin, S., van Dijk, I., & Valls, M. (2012). A luminescent reporter evidences active expression of Ralstonia solanacearum type III secretion system genes throughout plant infection. *Microbiology (United Kingdom)*, *158*(8), 2107–2116. https://doi.org/10.1099/mic.0.058610-0

Monteiro, F., Solé, M., Van Dijk, I., & Valls, M. (2012). A chromosomal insertion toolbox for promoter probing, mutant complementation, and pathogenicity studies in Ralstonia solanacearum. *Molecular Plant-Microbe Interactions*, *25*(4), 557–568. https://doi.org/10.1094/MPMI-07-11-0201

Montes, N., Alonso-Blanco, C., & García-Arenal, F. (2019). Cucumber mosaic virus infection as a potential selective pressure on arabidopsis thaliana populations. *PLoS Pathogens*. https://doi.org/10.1371/journal.ppat.1007810

Montes, N., Vijayan, V., & Pagán, I. (2020). Trade-offs between host tolerances to different pathogens in plant–virus interactions. *Virus Evolution*. https://doi.org/10.1093/ve/veaa019

Mori, Y., Inoue, K., Ikeda, K., Nakayashiki, H., Higashimoto, C., Ohnishi, K., Kiba, A., & Hikichi, Y. (2015). The vascular plant-pathogenic bacterium Ralstonia solanacearum produces biofilms required for its virulence on the surfaces of tomato cells adjacent to intercellular spaces. *Molecular Plant Pathology*, *17*(6), 890–902. https://doi.org/10.1111/mpp.12335

Morris, C. E., Bardin, M., Kinkel, L. L., Moury, B., Nicot, P. C., & Sands, D. C. (2009). Expanding the paradigms of plant pathogen life history and evolution of parasitic fitness beyond agricultural boundaries. *PLoS Pathogens*, *5*(12). https://doi.org/10.1371/journal.ppat.1000693

Morris, C. E., & Monier, J. M. (2003). The Ecological Significance of Biofilm Formation by Plant-Associated Bacteria. *Annual Review of Phytopathology*, *41*(February), 429–453. https://doi.org/10.1146/annurev.phyto.41.022103.134521

Morris, C. E., Monteil, C. L., & Berge, O. (2013). The life history of pseudomonas syringae: Linking agriculture to earth system processes. *Annual Review of Phytopathology*, *51*, 85–104. https://doi.org/10.1146/annurev-phyto-082712-102402

Mukaihara, T., Hatanaka, T., Nakano, M., & Oda, K. (2016). Ralstonia solanacearum type III effector RipAY is a glutathione-degrading enzyme that is activated by plant cytosolic thioredoxins and suppresses plant immunity. *MBio*, *7*(2). https://doi.org/10.1128/mBio.00359-16

Mur, L. A. J., Simpson, C., Kumari, A., Gupta, A. K., & Gupta, K. J. (2017). Moving nitrogen to the centre of plant defence against pathogens. *Annals of Botany*, *119*(5), 703–709. https://doi.org/10.1093/aob/mcw179

Murata, Y., Tamura, N., Nakaho, K., & Mukaihara, T. (2006). Mutations in the IrpE gene of Ralstonia solanacearum affects Hrp pili production and virulence. *Molecular Plant-Microbe Interactions*, *19*(8), 884–895. https://doi.org/10.1094/MPMI-19-0884

Nakaho, K., Hibino, H., & Miyagawa, H. (2000). Possible mechanisms limiting movement of

Ralstonia solanacearum in resistant tomato tissues. *Journal of Phytopathology*, *148*(3), 181–190. https://doi.org/10.1046/j.1439-0434.2000.00476.x

Narayanasamy, P. (2017). Biology and Infection Process of Bacterial and Phyoplasmal Pathogenss. In *Microbial Plant Pathogens: Detection and Management in Seeds and Propagules* (1st Editio, pp. 619–668). https://doi.org/10.1002/9781119195801.ch8

Neumann, S., Paveley, N. D., Beed, F. D., & Sylvester-Bradley, R. (2004). Nitrogen per unit leaf area affects the upper asymptote of Puccinia striiformis f.sp. tritici epidemics in winter wheat. *Plant Pathology*, *53*(6), 725–732. https://doi.org/10.1111/j.1365-3059.2004.01107.x

Ney, B., Bancal, M. O., Bancal, P., Bingham, I. J., Foulkes, J., Gouache, D., Paveley, N., & Smith, J. (2013). Crop architecture and crop tolerance to fungal diseases and insect herbivory. Mechanisms to limit crop losses. *European Journal of Plant Pathology*. https://doi.org/10.1007/s10658-012-0125-z

Niño-Liu, D. O., Ronald, P. C., & Bogdanove, A. J. (2006). Xanthomonas oryzae pathovars: Model pathogens of a model crop. *Molecular Plant Pathology*, *7*(5), 303–324. https://doi.org/10.1111/j.1364-3703.2006.00344.x

Nobori, T., Velásquez, A. C., Wu, J., Kvitko, B. H., Kremer, J. M., Wang, Y., He, S. Y., & Tsuda, K. (2018). Transcriptome landscape of a bacterial pathogen under plant immunity. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(13), E3055–E3064. https://doi.org/10.1073/pnas.1800529115

Noda, T., & Kaku, H. (1999). Growth of Xanthomonas oryzae pv. oryzae In Planta and in Guttation Fluid of Rice. *Japanese Journal of Phytopathology*, *65*(1), 9–14. https://doi.org/10.3186/jjphytopath.65.9

Noda, T., & Yamamoto, T. (2008). Reaction of Leersia grasses to Xanthomonas oryzae pv. oryzae collected from Japan and Asian countries. *Journal of General Plant Pathology*, *74*(5), 395–401. https://doi.org/10.1007/s10327-008-0118-0

Occhialini, A., Cunnac, S., Reymond, N., Genin, S., & Boucher, C. (2005). Genome-wide analysis of gene expression in Ralstonia solanacearum reveals that the hrpB gene acts as a regulatory switch controlling multiple virulence pathways. *Molecular Plant-Microbe Interactions*, *18*(9), 938–949. https://doi.org/10.1094/MPMI-18-0938

Oerke, E. C. (2006). Crop losses to pests. *Journal of Agricultural Science*, *144*(1), 31–43. https://doi.org/10.1017/S0021859605005708

Oerke, E. C., & Dehne, H. W. (2004). Safeguarding production - Losses in major crops and the role of crop protection. *Crop Protection*, *23*(4), 275–285. https://doi.org/10.1016/j.cropro.2003.10.001

Oh, E., & Jeon, B. (2014). Role of alkyl hydroperoxide reductase (AhpC) in the biofilm formation of Campylobacter jejuni. *PLoS ONE*, *9*(1). https://doi.org/10.1371/journal.pone.0087312

Olea, F., Pérez-García, A., Cantón, F. R., Rivera, M. E., Cañas, R., Ávila, C., Cazorla, F. M., Cánovas, F. M., & De Vicente, A. (2004). Up-regulation and localization of asparagine synthetase in tomato leaves infected by the bacterial pathogen Pseudomonas syringae. *Plant and Cell Physiology*, *45*(6), 770–780. https://doi.org/10.1093/pcp/pch092

Olmo, D., Nieto, A., Adrover, F., Urbano, A., Beidas, O., Juan, A., Marco-Noales, E., López, M. M., Navarro, I., Monterde, A., Montes-Borrego, M., Navas-Cortés, J. A., & Landa, B. B. (2010). First detection of Xylella fastidiosa infecting cherry (Prunus avium) and Polygala

myrtifolia plants, in Mallorca Island, Spain. *Plant Disease*, *9*(1), 76–99. https://doi.org/10.1558/jsrnc.v4il.24

Olsson, K. (1976). Experience of Brown Rot Caused by Pseudomonas solanacearum (Smith) Smith in Sweden. *EPPO Bulletin*, *6*(4), 199–207. https://doi.org/10.1111/j.1365-2338.1976.tb01546.x

Orgambide, G., Montrozier, H., Servin, P., Roussel, J., Trigalet-Demery, D., & Trigalet, A. (1991). High heterogeneity of the exopolysaccharides of Pseudomonas solanacearum strain GMI 1000 and the complete structure of the major polysaccharide. *Journal of Biological Chemistry*, *266*(13), 8312–8321.

Ou, S. H. (1985). *Rice Diseases* (S. Kew (ed.)). Commonwealth Agricultural Bureau.

Pagán, I., & García-Arenal, F. (2020). Tolerance of Plants to Pathogens: A Unifying View. *Annual Review of Phytopathology*, *58*(1), 1–20. https://doi.org/10.1146/annurev-phyto-010820-012749

Panmanee, W., & Hassett, D. J. (2009). Differential roles of OxyR-controlled antioxidant enzymes alkyl hydroperoxide reductase (AhpCF) and catalase (KatB) in the protection of Pseudomonas aeruginosa against hydrogen peroxide in biofilm vs. planktonic culture. *FEMS Microbiology Letters*, *295*(2), 238–244. https://doi.org/10.1111/j.1574-6968.2009.01605.x

Parsot, C., Hamiaux, C., & Page, A. L. (2003). The various and varying roles of specific chaperones in type III secretion systems. *Current Opinion in Microbiology*, *6*(1), 7–14. https://doi.org/10.1016/S1369-5274(02)00002-4

Patil, V. U., Girimalla, V., Sagar, V., Chauhan, R. S., & Chakrabarti, S. K. (2017). Genome sequencing of four strains of Phylotype I, II and IV of Ralstonia solanacearum that cause potato bacterial wilt in India. *Brazilian Journal of Microbiology*, *48*(2), 193–195. https://doi.org/10.1016/j.bjm.2016.10.016

Peeters, N., Carrère, S., Anisimova, M., Plener, L., Cazalé, A. C., & Genin, S. (2013). Repertoire, unified nomenclature and evolution of the Type III effector gene set in the Ralstonia solanacearum species complex. *BMC Genomics*, *14*(1). https://doi.org/10.1186/1471-2164-14-859

Peeters, N., Guidot, A., Vailleau, F., & Valls, M. (2013). Ralstonia solanacearum, a widespread bacterial plant pathogen in the post-genomic era. *Molecular Plant Pathology*, *14*(7), 651–662. https://doi.org/10.1111/mpp.12038

Pegg, G. F. (1985). Life in a black hole — the micro-environment of the vascular pathogen. *Transactions of the British Mycological Society*, *85*(1), IN1-20. https://doi.org/10.1016/s0007-1536(85)80151-0

Perrier, A., Peyraud, R., Rengel, D., Barlet, X., Lucasson, E., Gouzy, J., Peeters, N., Genin, S., & Guidot, A. (2016). Enhanced in planta Fitness through Adaptive Mutations in EfpR, a Dual Regulator of Virulence and Metabolic Functions in the Plant Pathogen Ralstonia solanacearum. *PLoS Pathogens*, *12*(12), 1–23. https://doi.org/10.1371/journal.ppat.1006044

Pierce, N. B. (1892). *The California Vine Disease: a preliminary report of investigations*. Washington, DC: Gov. Print. Off.

Planas-Marquès, M., Bernardo-Faura, M., Paulus, J., Kaschani, F., Kaiser, M., Valls, M., Van Der Hoorn, R. A. L., & Coll, N. S. (2018). Protease Activities Triggered by Ralstonia

solanacearum Infection in Susceptible and Tolerant Tomato Lines. *Molecular and Cellular Proteomics*, *17*(6), 1112–1125. https://doi.org/10.1074/mcp.RA117.000052

Planas-Marquès, M., Kressin, J. P., Kashyap, A., Panthee, D. R., Louws, F. J., Coll, N. S., & Valls, M. (2020). Four bottlenecks restrict colonization and invasion by the pathogen Ralstonia solanacearum in resistant tomato. *Journal of Experimental Botany*, *71*(6), 2157–2171. https://doi.org/10.1093/jxb/erz562

Popa, C., Li, L., Gil, S., Tatjer, L., Hashii, K., Tabuchi, M., Coll, N. S., Ariño, J., & Valls, M. (2016). The effector AWR5 from the plant pathogen Ralstonia solanacearum is an inhibitor of the TOR signalling pathway. *Scientific Reports*. https://doi.org/10.1038/srep27058

Poueymiro, M., & Genin, S. (2009). Secreted proteins from Ralstonia solanacearum: a hundred tricks to kill a plant. *Current Opinion in Microbiology*, *12*(1), 44–52. https://doi.org/10.1016/j.mib.2008.11.008

Pradhanang, P. M., Ji, P., Momol, M. T., Olson, S. M., Mayfield, J. L., & Jones, J. B. (2005). Application of acibenzolar-S-methyl enhances host resistance in tomato against Ralstonia solanacearum. *Plant Disease*, *89*(9), 989–993. https://doi.org/10.1094/PD-89-0989

Preston, G. M. (2000). Pseudomonas syringae pv. tomato: the right pathogen, of the right plant, at the right time. *Molecular Plant Pathology*, *1*(5), 263–275. https://doi.org/10.1046/j.1364-3703.2000.00036.x

Prior, P. (Philippe), & Fegan, M. (2005). *Bacterial wilt disease and the Ralstonia solanacearum species complex* (p. 510). https://espace.library.uq.edu.au/view/UQ:71427

Prior P. et al., 1998. (2013). *Bacterial Wilt Disease: Molecular and Ecological Aspects*. https://books.google.com/books?id=run7CAAAQBAJ&pgis=1

Puigvert, M., Guarischi-Sousa, R., Zuluaga, P., Coll, N. S., Macho, A. P., Setubal, J. C., & Valls, M. (2017). Transcriptomes of ralstonia solanacearum during root colonization of solanum commersonii. *Frontiers in Plant Science*, *8*(March). https://doi.org/10.3389/fpls.2017.00370

Puigvert, M., Solé, M., López-Garcia, B., Coll, N. S., Beattie, K. D., Davis, R. A., Elofsson, M., & Valls, M. (2019). Type III secretion inhibitors for the management of bacterial plant diseases. *Molecular Plant Pathology*, *20*(1), 20–32. https://doi.org/10.1111/mpp.12736

Purcell, A. H., & Saunders, S. R. (1999). Fate of Pierce's disease strains of Xylella fastidiosa in common riparian plants in California. *Plant Disease*, *83*(9), 825–830. https://doi.org/10.1094/PDIS.1999.83.9.825

Qian, Y. liang, Wang, X. sheng, Wang, D. zhou, Zhang, L. na, Zu, C. long, Gao, Z. liang, Zhang, H. jun, Wang, Z. yong, Sun, X. yong, & Yao, D. nian. (2013). The detection of QTLs controlling bacterial wilt resistance in tobacco (N. tabacum L.). *Euphytica*, *192*(2), 259–266. https://doi.org/10.1007/s10681-012-0846-2

R Team, C. (2017). R Core Team (2017). R: A language and environment for statistical computing. *R Found. Stat. Comput. Vienna, Austria. URL Http://Www. R-Project. Org/., Page R Foundation for Statistical Computing*.

Råberg, L. (2014). How to Live with the Enemy: Understanding Tolerance to Parasites. *PLoS Biology*, *12*(11). https://doi.org/10.1371/journal.pbio.1001989

Rainey, P. B., & Preston, G. M. (2000). In vivo expression technology strategies: Valuable tools for biotechnology. *Current Opinion in Biotechnology*, *11*(5), 440–444.

https://doi.org/10.1016/S0958-1669(00)00132-4

Read, A. F. (1994). The evolution of virulence. *Trends in Microbiology*, *2*(3), 2–5.

Remenant, B., Babujee, L., Lajus, A., Médigue, C., Prior, P., & Allen, C. (2012). Sequencing of K60, type strain of the major plant pathogen Ralstonia solanacearum. *Journal of Bacteriology*, *194*(10), 2742–2743. https://doi.org/10.1128/JB.00249-12

Remenant, B., C.-G., A., G., G., C., E., W., C., A., M., F., O., P., M., E., A., C., G., S., D., M., S., M., V., B., & C., M. (2010). Genomes of three tomato pathogens within the Ralstonia solanacearum species complex reveal significant evolutionary divergence. *BMC Genomics*, *11*(1), no pagination. http://www.biomedcentral.com/1471-2164/11/379%5Cnhttp://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed12&NEWS=N&AN=359176909

Remigi, P., Anisimova, M., Guidot, A., Genin, S., & Peeters, N. (2011). Functional diversification of the GALA type III effector family contributes to Ralstonia solanacearum adaptation on different plant hosts. *New Phytologist*, *192*(4), 976–987. https://doi.org/10.1111/j.1469-8137.2011.03854.x

Report, S. (2016). Update of a database of host plants of Xylella fastidiosa: 20 November 2015. *EFSA Journal*, *14*(2). https://doi.org/10.2903/j.efsa.2016.4378

Rivard, C. L., & Louws, F. J. (2008). Grafting to manage soilborne diseases in heirloom tomato production. *HortScience*, *43*(7), 2104–2111. https://doi.org/10.21273/hortsci.43.7.2104

Roberts, D. P., Denny, T. P., & Schell, M. A. (1988). Cloning of the egl gene of Pseudomonas solanacearum and analysis of its role in phytopathogenicity. *Journal of Bacteriology*, *170*(4), 1445–1451. https://doi.org/10.1128/jb.170.4.1445-1451.1988

Roberts, M. G., & Heesterbeek, J. A. P. (2020). Characterizing reservoirs of infection and the maintenance of pathogens in ecosystems. *Journal of the Royal Society Interface*, *17*(162). https://doi.org/10.1098/rsif.2019.0540

Robinette, D., & Matthysse, A. G. (1990). Inhibition by Agrobacterium tumefaciens and Pseudomonas savastanoi of development of the hypersensitive response elicited by Pseudomonas syringae pv. phaseolicola. *Journal of Bacteriology*, *172*(10), 5742–5749. https://doi.org/10.1128/jb.172.10.5742-5749.1990

Roine, E., Raineri, D. M., Romantschuk, M., Wilson, M., & Nunn, D. N. (1998). Characterization of type IV pilus genes in Pseudomonas syringae pv. tomato DC3000. *Molecular Plant-Microbe Interactions*, *11*(11), 1048–1056. https://doi.org/10.1094/MPMI.1998.11.11.1048

Romantschuk, M. (1992). Attachment of Plant Pathogenic Bacteria to Plant Surfaces. *Annu. Rev. Phytopathol.*, *30*, 225–243.

Rooney, J. M., & Hoad, G. V. (1989). Compensation in growth and photosynthesis of wheat (Triticum aestivum L.) following early inoculations with Septoria nodorum (Berk.) Berk. *New Phytologist*. https://doi.org/10.1111/j.1469-8137.1989.tb00363.x

Saile, E., McGarvey, J. A., Schell, M. A., & Denny, T. P. (1997). Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by Ralstonia solanacearum. *Phytopathology*, *87*(12), 1264–1271. https://doi.org/10.1094/PHYTO.1997.87.12.1264

Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A.,

Brottiert, P., Camus, J. C., Cattolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., … Boucher, C. A. (2002). Genome sequence of the plant pathogen ralstonia solanacearum. *Nature*, *415*(6871), 497–502. https://doi.org/10.1038/415497a

Sampedro, I., Parales, R. E., Krell, T., & Hill, J. E. (2015). Pseudomonas chemotaxis. *FEMS Microbiology Reviews*, *39*(1), 17–46. https://doi.org/10.1111/1574-6976.12081

Sands, D. C., Langhans, V. E., Scharen, A. L., & Smet, G. de. (1982). The association between bacteria and rain and possible resutant meteorological implications. *Joournal of the Hungariun Meteorological Service*, *86*(2), 148–152.

Sang, Y., Wang, Y., Ni, H., Cazalé, A. C., She, Y. M., Peeters, N., & Macho, A. P. (2018). The ralstonia solanacearum type III effector ripay targets plant redox regulators to suppress immune responses. *Molecular Plant Pathology*, *19*(1), 129–142. https://doi.org/10.1111/mpp.12504

Saponari, M., Boscia, D., Nigro, F., & Martelli, G. P. (2013). Identification of dna sequences related to Xylella fastidiosa in oleander, almond and olive trees exhibiting leaf scorch symptoms in Apulia (Southern Italy). *Journal of Plant Pathology*, *95*(3), 668. https://doi.org/10.4454/JPP.V95I3.035

Schaad, N. W. (2008). Emerging Plant Pathogenic Bacteria and Global Warming. In *Pseudomonas syringae Pathovars and Related Pathogens - Identification, Epidemiology and Genomics* (pp. 369–379).

Schell, M. A., Roberts, D. P., & Denny, T. P. (1988). Analysis of the Pseudomonas solanacearum polygalacturonase encoded by pglA and its involvement in phytopathogenicity. *Journal of Bacteriology*, *170*(10), 4501–4508. https://doi.org/10.1128/jb.170.10.4501-4508.1988

Schell, Mark A. (2000). Control of virulence and pathogenicity genes of Ralstonia solanacearum by an elaborate sensory network. *Ann. Rev. Phytopathol*, 263–284.

Schneider, R. W. (1977). Bacterial Speck of Tomato: Sources of Inoculum and Establishment of a Resident Population. *Phytopathology*, *77*(3), 388. https://doi.org/10.1094/phyto-67-388

Scholthof, K. B. (2007). The disease triangle: pathogens, the environment and society. *Nature Reviews Microbiology*, *5*(2), 152–156. https://doi.org/10.1038/nrmicro1596

Sequeira, L., & Rowe, P. R. (1969). Selection and utilization of solanum phureja clones with high resistance to different strains of pseudomonas solanacearum. *American Potato Journal*, *46*(12), 451–462. https://doi.org/10.1007/BF02862028

Shamsuddin, N., Lloyd, A. B., & Graham, J. (1978). Survival of the potato strain of Pseudomonas solanacearum in soil. *Journal Australian Institute of Agricultural Science*, *44*(3–4), 212–215.

Shinohara, R., Kanda, A., Ohnishi, K., Kiba, A., & Hikichi, Y. (2005). Contribution of folate biosynthesis to Ralstonia solanacearum proliferation in intercellular spaces. *Applied and Environmental Microbiology*, *71*(1), 417–422. https://doi.org/10.1128/AEM.71.1.417-422.2005

Shrivastava, S., & Mande, S. S. (2008). Identification and functional characterization of gene components of type VI secretion system in bacterial genomes. *PLoS ONE*, *3*(8). https://doi.org/10.1371/journal.pone.0002955

Sicard, A., Zeilinger, A. R., Vanhove, M., Schartel, T. E., Beal, D. J., Daugherty, M. P., & Almeida, R. P. P. (2018). Xylella fastidiosa: Insights into an emerging plant pathogen. *Annual Review of Phytopathology*, *56*(June), 181–202. https://doi.org/10.1146/annurev-phyto-080417-045849

Simpson, A. J. G., Reinach, F. C., Arruda, P., Abreu, F. A., Acencio, M., Alvarenga, R., Alves, L. M. C., Araya, J. E., Baia, G. S., Baptista, C. S., Barros, M. H., Bonaccorsi, E. D., Bordin, S., Bové, J. M., Briones, M. R. S., Bueno, M. R. P., Camargo, A. A., Camargo, L. E. A., Carraro, D. M., … Setubal, J. C. (2000). The genome sequence of the plant pathogen Xylella fastidiosa: The Xylella fastidiosa consortium of the organization for nucleotide sequencing and analysis, Sao Paulo, Brazil. *Nature*, *406*(6792), 151–157. https://doi.org/10.1038/35018003

Siri, M. I., Sanabria, A., & Pianzzola, M. J. (2011). Genetic diversity and aggressiveness of Ralstonia solanacearum strains causing bacterial wilt of potato in Uruguay. *Plant Disease*, *95*(10), 1292–1301. https://doi.org/10.1094/PDIS-09-10-0626

Smith, E. F. (1896). A bacterial disease of the tomato, eggplant, and Irish potato (Bacillus solanacearum n. sp.). *USDA Bulleting Vegetable Pathology*, *12*, 34.

Solé, M., Popa, C., Mith, O., Sohn, K. H., Jones, J. D. G., Deslandes, L., & Valls, M. (2012). Type III Effectors Displaying Virulence and Avirulence Activities. *Molecular Plant-Microbe Interactions*, *25*(7), 941–953. https://doi.org/10.1094/MPMI-12-11-0321

Steck, T. R. (2001). The Viable But Nonculturable State of. *Society*, *67*(9), 3866–3872. https://doi.org/10.1128/AEM.67.9.3866

Strange, R. N., & Scott, P. R. (2005). Plant Disease: A Threat to Global Food Security. *Annual Review of Phytopathology*, *43*(1), 83–116. https://doi.org/10.1146/annurev.phyto.43.113004.133839

Sun, Y., Wang, K., Caceres-Moreno, C., Jia, W., Chen, A., Zhang, H., Liu, R., & Macho, A. P. (2017). Genome sequencing and analysis of Ralstonia solanacearum phylotype I strains FJAT-91, FJAT-452 and FJAT-462 isolated from tomato, eggplant, and chili pepper in China. *Standards in Genomic Sciences*, *12*(1), 1–11. https://doi.org/10.1186/s40793-017-0241-7

Sundin, G. W., Castiblanco, L. F., Yuan, X., Zeng, Q., & Yang, C. H. (2016). Bacterial disease management: Challenges, experience, innovation and future prospects: Challenges in bacterial molecular plant pathology. *Molecular Plant Pathology*, *17*(9), 1506–1518. https://doi.org/10.1111/mpp.12436

Swings, J., Van Den Mooter, M., Vauterin, L., Hoste, B., Gillis, M., Mew, T. W., & Kersters, K. (1990). Reclassification of the causal agents of bacterial blight (Xanthomonas campestris pv. oryzae) and bacterial leaf streak (Xanthomonas campestris pv. oryzicola) of rice as pathovars of Xanthomonas oryzae (ex Ishiyama 1922) sp. nov., nom. rev. *International Journal of Systematic Bacteriology*, *40*(3), 309–311. https://doi.org/10.1099/00207713-40-3-309

Tans-Kersten, J., Brown, D., & Allen, C. (2004). Swimming motility, a virulence trait of Ralstonia solanacearum, is regulated by FlhDC and the plant host environment. *Molecular Plant-Microbe Interactions*, *17*(6), 686–695. https://doi.org/10.1094/MPMI.2004.17.6.686

Tans-Kersten, J., Guan, Y., & Allen, C. (1998). Ralstonia solanacearum pectin methylesterase is required for growth on methylated pectin but not for bacterial wilt virulence. *Applied and Environmental Microbiology*, *64*(12), 4918–4923.

https://doi.org/10.1128/aem.64.12.4918-4923.1998

Tans-kersten, J., Huang, H., Allen, C., Tans-kersten, J., Huang, H., & Allen, C. (2001). Ralstonia solanacearum Needs Motility for Invasive Virulence on Tomato Ralstonia solanacearum Needs Motility for Invasive Virulence on Tomato Downloaded from http://jb.asm.org/ on February 6 , 2013 by University of Pretoria : Academic Information Service. *Journal of Bacteriology*, *183*(12), 3597–3605. https://doi.org/10.1128/JB.183.12.3597

Tapsoba, H., & Wilson, J. P. (1997). Effects of temperature and light on germination of urediniospores of the pearl millet rust pathogen, Puccinia substriata var. indica. *Plant Disease*, *81*(9), 1049–1052. https://doi.org/10.1094/PDIS.1997.81.9.1049

Thomas, N. A., Ma, I., Prasad, M. E., & Rafuse, C. (2012). Expanded roles for multicargo and class 1B effector chaperones in type III secretion. *Journal of Bacteriology*, *194*(15), 3767–3773. https://doi.org/10.1128/JB.00406-12

Torto-Alalibo, T., Collmer, C. W., & Gwinn-Giglio, M. (2009). The Plant-Associated Microbe Gene Ontology (PAMGO) Consortium: Community development of new Gene Ontology terms describing biological processes involved in microbe-host interactions. *BMC Microbiology*, *9*(SUPPL. 1). https://doi.org/10.1186/1471-2180-9-S1-S1

Torto-Alalibo, T., Collmer, C. W., Gwinn-Giglio, M., Lindeberg, M., Meng, S., Chibucos, M. C., Tseng, T.-T., Lomax, J., Biehl, B., Ireland, A., Bird, D., Dean, R. A., Glasner, J. D., Perna, N., Setubal, J. C., Collmer, A., & Tyler, B. M. (2010). Unifying Themes in Microbial Associations with Animal and Plant Hosts Described Using the Gene Ontology. *Microbiology and Molecular Biology Reviews*, *74*(4), 479–503. https://doi.org/10.1128/mmbr.00017-10

Tran, T. M., MacIntyre, A., Hawes, M., & Allen, C. (2016). Escaping Underground Nets: Extracellular DNases Degrade Plant Extracellular Traps and Contribute to Virulence of the Plant Pathogenic Bacterium Ralstonia solanacearum. *PLoS Pathogens*, *12*(6), 1–26. https://doi.org/10.1371/journal.ppat.1005686

Tsujimoto, S., Nakaho, K., Adachi, M., Ohnishi, K., Kiba, A., & Hikichi, Y. (2008). Contribution of the type II secretion system in systemic infectivity of Ralstonia solanacearum through xylem vessels. *Journal of General Plant Pathology*, *74*(1), 71–75. https://doi.org/10.1007/s10327-007-0061-5

Tusiime, G., Adipala, E., Opio, F., & Bhagsari, A. S. (1997). Weeds as latent hosts of Ralstonia solanacearum in highland Uganda: implications to development of an integrated control package for bacterial wilt. In *Bacterial Wilt Disease: Molecular and Ecological Aspects.* (pp. 413–419).

Tzfira, T., & Citovsky, V. (2002). Partners-in-infection: Host proteins involved in the transformation of plant cells by Agrobacterium. *Trends in Cell Biology*, *12*(3), 121–129. https://doi.org/10.1016/S0962-8924(01)02229-2

Uppalapati, S. R., Ishiga, Y., Wangdi, T., Urbanczyk-Wochniak, E., Ishiga, T., Mysore, K. S., & Bender, C. L. (2008). Pathogenicity of Pseudomonas syringae pv. tomato on tomato seedlings: Phenotypic and gene expression analyses of the virulence function of coronatine. *Molecular Plant-Microbe Interactions*, *21*(4), 383–395. https://doi.org/10.1094/MPMI-21-4-0383

Uwamahoro, F., Berlin, A., Bucagu, C., Bylund, H., & Yuen, J. (2020). Ralstonia solanacearum causing potato bacterial wilt: host range and cultivars' susceptibility in Rwanda. *Plant Pathology*, *69*(3), 559–568. https://doi.org/10.1111/ppa.13140

Valls, M., Genin, S., & Boucher, C. (2006). Integrated regulation of the type III secretion system and other virulence determinants in Ralstonia solanacearum. *PLoS Pathogens*, *2*(8), 0798–0807. https://doi.org/10.1371/journal.ppat.0020082

Van Attikum, H., Bundock, P., & Hooykaas, P. J. J. (2001). Non-homologous end-joining proteins are required for Agrobacterium T-DNA integration. *EMBO Journal*, *20*(22), 6550–6558. https://doi.org/10.1093/emboj/20.22.6550

Van Dam, N. M. (2009). How plants cope with biotic interactions. *Plant Biology*, *11*(1), 1–5. https://doi.org/10.1111/j.1438-8677.2008.00179.x

Van Den Bosch, F., Akudibilah, G., Seal, S., & Jeger, M. (2006). Host resistance and the evolutionary response of plant viruses. *Journal of Applied Ecology*, *43*(3), 506–516. https://doi.org/10.1111/j.1365-2664.2006.01159.x

Van Elsas, J. D., Kastelein, P., Van Bekkum, P., Van der Wolf, J. M., De Vries, P. M., & Van Overbeek, L. S. (2000). Survival of Ralstonia solanacearum biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. *Phytopathology*, *90*(12), 1358–1366. https://doi.org/10.1094/PHYTO.2000.90.12.1358

van Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P., & Boucher, C. (1995). The hrp gene locus of Pseudomonas solanacearum, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. *Molecular Microbiology*, *15*(6), 1095–1114. https://doi.org/10.1111/j.1365-2958.1995.tb02284.x

Vasse, J., Frey, P., & Trigalet, A. (1995). Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by Pseudomonas solanacearum. *Molecular Plant-Microbe Interactions*, *8*(2), 241–251. https://doi.org/10.1094/MPMI-8-0241

Vasse, Jacques, Genin, S., Frey, P., Boucher, C., & Brito, B. (2000). The hrpB and hrpG regulatory genes of Ralstonia solanacearum are required for different stages of the tomato root infection process. *Molecular Plant-Microbe Interactions*, *13*(3), 259–267. https://doi.org/10.1094/MPMI.2000.13.3.259

Wairuri, C. K., Van Der Waals, J. E., Van Schalkwyk, A., & Theron, J. (2012). Ralstonia solanacearum needs Flp pili for virulence on potato. *Molecular Plant-Microbe Interactions*, *25*(4), 546–556. https://doi.org/10.1094/MPMI-06-11-0166

Wang, M. N., Wan, A. M., & Chen, X. M. (2015). Barberry as alternate host is important for puccinia graminis f. Sp. Tritici but not for puccinia striiformis f. Sp. Tritici in the U.S. Pacific Northwest. *Plant Disease*, *99*(11), 1507–1516. https://doi.org/10.1094/PDIS-12-14-1279-RE

Wang, W. X., Barak, T., Vinocur, B., Shoseyov, O., & Altman, A. (2003). Abiotic resistance and chaperones: possible physiological role of SP1, a stable and stabilizing protein from Populus. In *Plant biotechnology 2000 and beyond* (pp. 439–443).

Wasim, M., Bible, A. N., Xie, Z., & Alexandre, G. (2009). Alkyl hydroperoxide reductase has a role in oxidative stress resistance and in modulating changes in cell-surface properties in Azospirillum brasilense Sp245. *Microbiology*, *155*(4), 1192–1202. https://doi.org/10.1099/mic.0.022541-0

Waugh, M. M., Kim, D. H., Ferrin, D. M., & Stanghellini, M. E. (2003). Reproductive potential of Monosporascus cannonballus. *Plant Disease*, *87*(1), 45–50. https://doi.org/10.1094/PDIS.2003.87.1.45

Weese, T. L., & Bohs, L. (2007). A three-gene phylogeny of the genus Solanum (Solanaceae). *Systematic Botany*, *32*(2), 445–463. https://doi.org/10.1600/036364407781179671

Wei, Y., Sang, Y., & Macho, A. P. (2017). The ralstonia solanacearum type III effector RipAY is phosphorylated in plant cells to modulate its enzymatic activity. *Frontiers in Plant Science*, *8*(November), 1–7. https://doi.org/10.3389/fpls.2017.01899

Wells, J. M., Raju, B. C., Hung, H.-Y., Weisburg, W. G., Mandelco-Paul, L., & Brenner, D. J. (1987). Xylella fastidiosa gen. nov., sp. nov: Gram-Negative, Xylem-Limited, Fastidious Plant Bacteria Related to Xanthomonas spp. *International Journal of Systematic Bacteriology*, *37*(2), 136–143. https://doi.org/10.1099/00207713-37-2-136

Wenneker, M., Verdel, M. S. W., Groeneveld, R. M. W., Kempenaar, C., Van Beuningen, A. R., & Janse, J. D. (1999). Ralstonia (Pseudomonas) solanacearum race 3 (biovar 2) in surface water and natural weed hosts: First report on stinging nettle (Urtica dioica). *European Journal of Plant Pathology*, *105*(3), 307–315. https://doi.org/10.1023/A:1008795417575

Wessel, A. K., Arshad, T. A., Fitzpatrick, M., Connell, J. L., Bonnecaze, R. T., Shear, J. B., & Whiteley, M. (2014). Oxygen limiation within a bacterial aggregate. *MBio*, *5*(2), 1–9. https://doi.org/10.1128/mBio.00992-14.Editor

Whalen, M. C., Innes, R. W., Bent, A. F., & Staskawicz, B. J. (1991). Identification of Pseudomonas syringae pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. *Plant Cell*, *3*(1), 49–59. https://doi.org/10.1105/tpc.3.1.49

Winans, S. C. (1992). Two-Way Chemical Signaling in Agrobacterium-Plant Interactions. *Microbiological Reviews*, *56*(1), 12–31.

Xian, L., Yu, G., Wei, Y., Rufian, J. S., Li, Y., Zhuang, H., Xue, H., Morcillo, R. J. L., & Macho, A. P. (2020). A Bacterial Effector Protein Hijacks Plant Metabolism to Support Pathogen Nutrition. *Cell Host and Microbe*, 1–10. https://doi.org/10.1016/j.chom.2020.07.003

Xin, X. F., & He, S. Y. (2013). Pseudomonas syringae pv. tomato DC3000: A model pathogen for probing disease susceptibility and hormone signaling in plants. *Annual Review of Phytopathology*, *51*, 473–498. https://doi.org/10.1146/annurev-phyto-082712-102321

Xu, J., Zheng, H. J., Liu, L., Pan, Z. C., Prior, P., Tang, B., Xu, J. S., Zhang, H., Tian, Q., Zhang, L. Q., & Feng, J. (2011). Complete genome sequence of the plant pathogen Ralstonia solanacearum strain Po82. *Journal of Bacteriology*, *193*(16), 4261–4262. https://doi.org/10.1128/JB.05384-11

Yao, J., & Allen, C. (2006). Chemotaxis is required for virulence and competitive fitness of the bacterial wilt pathogen Ralstonia solanacearum. *Journal of Bacteriology*, *188*(10), 3697–3708. https://doi.org/10.1128/JB.188.10.3697-3708.2006

Yoshimochi, T., Hikichi, Y., Kiba, A., & Ohnishi, K. (2009). The global virulence regulator PhcA negatively controls the Ralstonia solanacearum hrp regulatory cascade by repressing expression of the PrhIR signaling proteins. *Journal of Bacteriology*, *191*(10), 3424–3428. https://doi.org/10.1128/JB.01113-08

Yu, G., Wang, L. G., Han, Y., & He, Q. Y. (2012). ClusterProfiler: An R package for comparing biological themes among gene clusters. *OMICS A Journal of Integrative Biology*, *16*(5), 284–287. https://doi.org/10.1089/omi.2011.0118

Yu, J., Penaloza-Vázquez, A., Chakrabarty, A. M., & Bender, C. L. (1999). Involvement of the exopolysaccharide alginate in the virulence and epiphytic fitness of Pseudomonas

syringae pv. syringae. *Molecular Microbiology*, *33*(4), 712–720. https://doi.org/10.1046/j.1365-2958.1999.01516.x

Yu, X., Lund, S. P., Scott, R. A., Greenwald, J. W., Records, A. H., Nettleton, D., Lindow, S. E., Gross, D. C., & Beattie, G. A. (2013). Transcriptional responses of Pseudomonas syringae to growth in epiphytic versus apoplastic leaf sites. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(5). https://doi.org/10.1073/pnas.1221892110

Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M., & Schell, J. (1983). Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *The EMBO Journal*, *2*(12), 2143–2150. https://doi.org/10.1002/j.1460-2075.1983.tb01715.x

Zaumeyer, W. J. (1958). Antibiotics in the control of plant diseases. *Annual Review of Microbiology*, *12*, 415–440.

Zhang, L., Xu, J., Xu, J., Chen, K., He, L., & Feng, J. (2012). TssM is essential for virulence and required for type VI secretion in Ralstonia solanacearum. In *Journal of Plant Diseases and Protection* (Vol. 119, Issue 4).

Zhang, L., Xu, J., Xu, J., Zhang, H., He, L., & Feng, J. (2014). TssB is essential for virulence and required for Type VI secretion system in Ralstonia solanacearum. *Microbial Pathogenesis*, *74*(1), 1–7. https://doi.org/10.1016/j.micpath.2014.06.006

Zhao, Jian, Devaiah, S. P., Wang, C., Li, M., Welti, R., & Wang, X. (2013). Arabidopsis phospholipase Dβ1 modulates defense responses to bacterial and fungal pathogens. *New Phytologist*. https://doi.org/10.1111/nph.12256

Zhao, Jie, Wang, M., Chen, X., & Kang, Z. (2016). Role of Alternate Hosts in Epidemiology and Pathogen Variation of Cereal Rusts. *Annual Review of Phytopathology*, *54*, 207–228. https://doi.org/10.1146/annurev-phyto-080615-095851

Zhu, J., Oger, P. M., Schrammeijer, B., Hooykaas, P. J. J., Farrand, S. K., & Winans, S. C. (2000). The bases of crown gall tumorigenesis. *Journal of Bacteriology*, *182*(14), 3885–3895. https://doi.org/10.1128/JB.182.14.3885-3895.2000

Ziemienowicz, A., Tinland, B., Bryant, J., Gloeckler, V., & Hohn, B. (2000). Plant Enzymes but Not AgrobacteriumVirD2 Mediate T-DNA Ligation In Vitro. *Molecular and Cellular Biology*, *20*(17), 6317–6322. https://doi.org/10.1128/mcb.20.17.6317-6322.2000

Zuleta, M. C. (n.d.). *Identification of Countermeasures Identification of Countermeasures*.

Zuluaga, A. P., Puigvert, M., & Valls, M. (2013). Novel plant inputs influencing Ralstonia solanacearum during infection. *Frontiers in Microbiology*, *4*(NOV), 349. https://doi.org/10.3389/fmicb.2013.00349

ANNEX

RESEARCH ARTICLE
Host-Microbe Biology

Twitching and Swimming Motility Play a Role in *Ralstonia solanacearum* Pathogenicity

Jordi Corral,[a] Pau Sebastià,[b] Núria S. Coll,[b] Jordi Barbé,[a] Jesús Aranda,[a] Marc Valls[b,c]

[a]Departament de Genètica i Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Cerdanyola del Vallès (Barcelona), Catalonia, Spain
[b]Centre for Research in Agricultural Genomics (CSIC-IRTA-UAB-UB), Cerdanyola del Vallès (Barcelona), Catalonia, Spain
[c]Genetics Section, Universitat de Barcelona, Barcelona, Catalonia, Spain

Jesús Aranda and Marc Valls contributed equally to this article as senior authors. Author order was determined on the basis of seniority.

ABSTRACT *Ralstonia solanacearum* is a bacterial plant pathogen causing important economic losses worldwide. In addition to the polar flagella responsible for swimming motility, this pathogen produces type IV pili (TFP) that govern twitching motility, a flagellum-independent movement on solid surfaces. The implication of chemotaxis in plant colonization, through the control flagellar rotation by the proteins CheW and CheA, has been previously reported in *R. solanacearum*. In this work, we have identified in this bacterium homologues of the *Pseudomonas aeruginosa pilI* and *chpA* genes, suggested to play roles in TFP-associated motility analogous to those played by the *cheW* and *cheA* genes, respectively. We demonstrate that *R. solanacearum* strains with a deletion of the *pilI* or the *chpA* coding region show normal swimming and chemotaxis but altered biofilm formation and reduced twitching motility, transformation efficiency, and root attachment. Furthermore, these mutants displayed wild-type growth *in planta* and impaired virulence on tomato plants after soil-drench inoculations but not when directly applied to the xylem. Comparison with deletion mutants for *pilA* and *fliC*—encoding the major pilin and flagellin subunits, respectively—showed that both twitching and swimming are required for plant colonization and full virulence. This work proves for the first time the functionality of a pilus-mediated pathway encoded by *pil-chp* genes in *R. solanacearum*, demonstrating that *pilI* and *chpA* genes are bona fide motility regulators controlling twitching motility and its three related phenotypes: virulence, natural transformation, and biofilm formation.

IMPORTANCE Twitching and swimming are two bacterial movements governed by pili and flagella. The present work identifies for the first time in the Gram-negative plant pathogen *Ralstonia solanacearum* a pilus-mediated chemotaxis pathway analogous to that governing flagellum-mediated chemotaxis. We show that regulatory genes in this pathway control all of the phenotypes related to pili, including twitching motility, natural transformation, and biofilm formation, and are also directly implicated in virulence, mainly during the first steps of the plant infection. Our results show that pili have a higher impact than flagella on the interaction of *R. solanacearum* with tomato plants and reveal new types of cross-talk between the swimming and twitching motility phenotypes: enhanced swimming in bacteria lacking pili and a role for the flagellum in root attachment.

KEYWORDS *Ralstonia solanacearum*, *pilI*, *chpA*, *pilA*, *fliC*

Ralstonia solanacearum is a soilborne Gram-negative bacterium that causes a plant disease known as bacterial wilt mainly in tropical and subtropical climates (1). *R. solanacearum* exhibits an unusually broad host range comprising more than 200 plant

Citation Corral J, Sebastià P, Coll NS, Barbé J, Aranda J, Valls M. 2020. Twitching and swimming motility play a role in *Ralstonia solanacearum* pathogenicity. mSphere 5:e00740-19. https://doi.org/10.1128/mSphere.00740-19.

Editor Grant R. Bowman, University of Wyoming

Address correspondence to Marc Valls, marcvalls@ub.edu.

Received 14 October 2019
Accepted 19 February 2020
Published 4 March 2020

species from over 50 families, including potato, tomato, tobacco, peanut, and banana, among other crops (2). These facts have contributed to the ranking of *R. solanacearum* as among of the most destructive plant-pathogenic bacterial species worldwide (3).

Plant colonization begins with the attachment of *R. solanacearum* on roots and entry into the host plant through wounds, at sites of secondary root emergence or elongation (4). The bacterium subsequently colonizes the root cortex and moves to the xylem, where it spreads systematically, grows extensively, and produces large amounts of exopolysaccharides (EPS) that cause vascular obstruction. This blockage results in wilting of the stem and leaves and, eventually, plant death (1).

In order to reach different plant tissues and get inside the vascular system, *R. solanacearum* uses different types of movement strategies. The first is swimming motility, an individual cell movement powered by rotating flagella and produced in aqueous environments. In *R. solanacearum*, this kind of motility is mediated by one to four polar flagella and mutants lacking either FliC (the flagellar subunit protein) or FliM (the flagellar motor switch protein) are nonmotile and present a reduction of virulence in tomato after soil-soak inoculation (5). Chemotaxis enables bacterial cells to sense specific chemicals and depends on the presence of several proteins, which ultimately interact with the flagellar motor to move toward more-favorable conditions. This complex behavior begins in cell membrane-associated receptors, called MCPs (methyl-accepting chemotaxis proteins), which detect environmental stimuli and respond to them by changing their conformation. These changes trigger autophosphorylation of the cytoplasmic histidine autokinase CheA, which forms a complex with the receptor through the coupling protein CheW (Fig. 1A). CheA transfers its phosphate group to CheY, a diffusible cytoplasmic response regulator that interacts with the flagellar motor to switch its direction of rotation. Both *R. solanacearum cheA* and *cheW* null mutants are motile but nonchemotactic, and their virulence is as low as that of a completely nonmotile *fliC* knockout mutant (6).

The other movement used by *R. solanacearum* is twitching motility, a coordinated multicellular movement driven by the extension, attachment, and retraction of the type IV pilus (TFP) appendages in solid surfaces or viscous media. In Gram-negative bacteria, the TFP system requires at least 35 *pil* genes for the synthesis, display, and function of polar and retractable TFP (7). *R. solanacearum* also possesses TFP-mediated motility, which plays a role in natural transformation, biofilm formation, and virulence (8). The genes *pilA*, *pilQ*, and *pilT*, whose products are a monomer of the major pilin protein, the secretin involved in the pilus extrusion, and the protein required for pilus retraction, respectively, have been identified in *R. solanacearum*, and inactivation of any of them reduces both twitching motility and virulence (8, 9). In addition, the *pilA* mutant was reduced in virulence on tomato plants, in attachment to roots, and in biofilm formation as well as being not naturally competent for transformation (8).

In the Gram-negative nosocomial pathogen *Pseudomonas aeruginosa*, which bears both flagella and pili, a hypothetical pilus-mediated chemotaxis pathway encoded by the *pil-chp* genes in so-called cluster IV has been proposed to exist based on homology to the flagellar chemotaxis system (10, 11). In a manner analogous to that seen with flagellum-mediated chemotaxis, in this pathway the molecular signal generated by the cell membrane-associated receptor (PilJ) is expected to trigger autophosphorylation of the cytoplasmic CheA-like histidine autokinase called ChpA, which may form a complex with two CheW homologues called PilI and ChpC (Fig. 1A). The control of movement of pili in this hypothetical system in *P. aeruginosa* is likely performed by two CheY homologues (PilG and PilH), which would interact with the putative TFP motor to control twitching motility after their phosphorylation by ChpA (10, 11).

In this report, we describe a new gene cluster in *R. solanacearum* with strong similarities to *P. aeruginosa* cluster IV, including genes encoding PilI and ChpA homologues. We have constructed *pilI* and *chpA* knockout mutants and mutants in well-described twitching (*pilA*) and swimming (*fliC*) motility genes in *R. solanacearum* and have studied the role of these genes in *R. solanacearum* motility and plant colonization

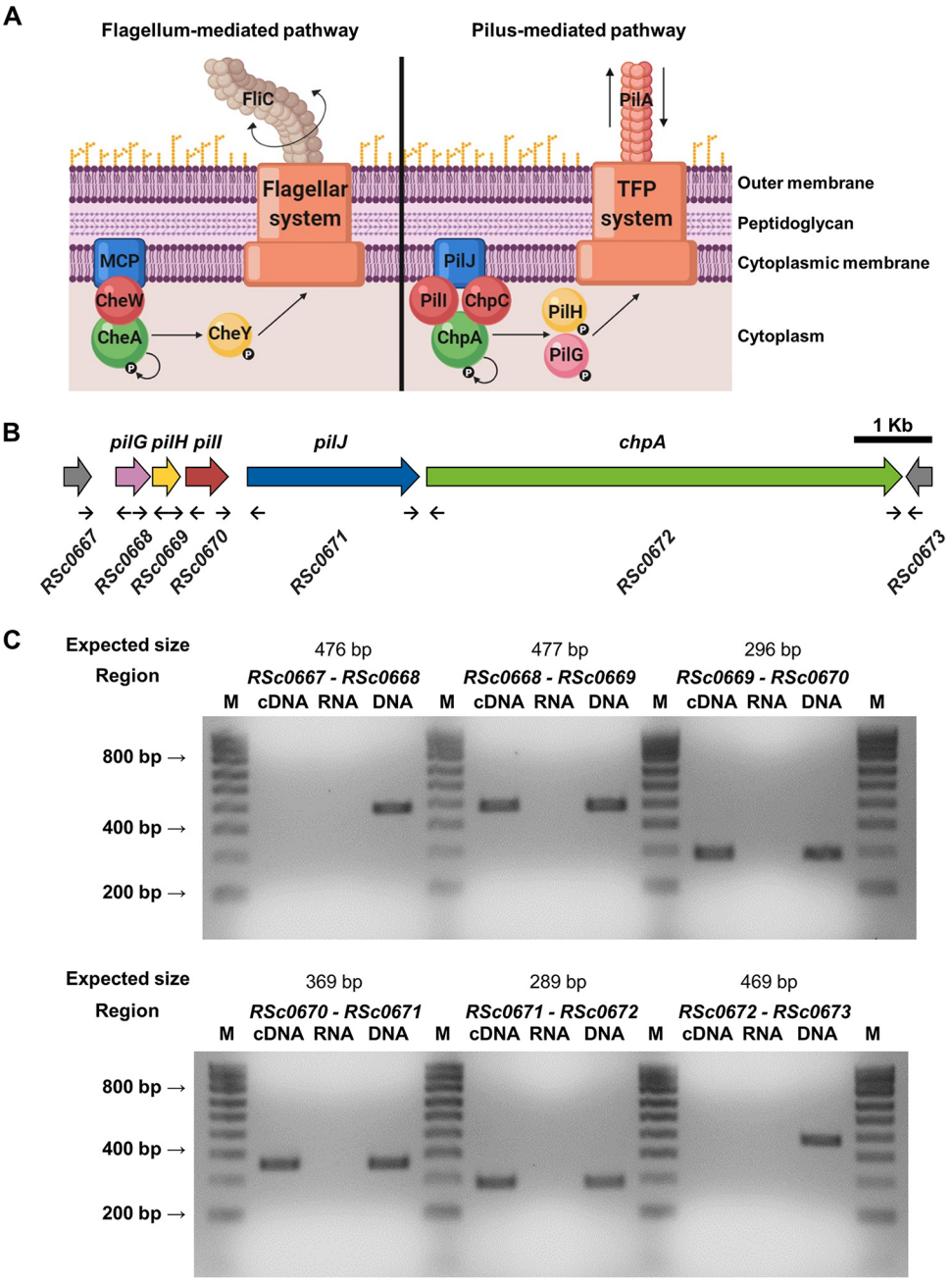


FIG 1 Characterization of the *pil-chp* operon. (A) Representation of flagellin-dependent (left) and pilus-dependent (right) pathways based on protein homology data from Sampedro et al. (11). (B) Schematic diagram of the *pil-chp* operon gene cluster in the *R. solanacearum* GMI1000 genome. Small arrows represent the oligonucleotides used in the RT-PCR. Spacings between protein-coding sequences in the *pil-chp* operon are as follows: *pilG-pilH* 31 bp, *pilH-pilI* 58 bp, *pilI-pilJ* 245 bp, *pilJ-chpA* 79 bp. (C) RT-PCRs of the indicated *pil-chp* intergenic regions. Each primer pair (Table S1) was used to prepare a PCR mixture with cDNA, RNA, or DNA from the GMI1000 strain as a template. V Ladder NzyTech was used as the DNA marker (M).

and in other related processes such as chemotaxis, biofilm formation, and natural transformation.

RESULTS

Analysis of the *R. solanacearum* GMI1000 genome reveals the presence of single *pilI* (CheW-like) and *chpA* (CheA-like) orthologues clustered in a *pil-chp* operon. Orthologous analysis revealed that both TFP-associated protein domains, the CheW-like and CheA-like domains, were found in two genes located in a putative

operon in the GMI1000 genome sequence, which includes in total five *pil-chp* homologues: *R. solanacearum RSc0668* (*pilG*), *RSc0669* (*pilH*), *RSc0670* (*pilI*), *RSc0671* (*pilJ*), and *RSc0672* (*chpA*) (Fig. 1B). This cluster is syntenic to that previously described in *P. aeruginosa*, except that it lacks the *chpB* and *chpC* genes downstream of *chpA*. CheW-like and CheA-like domains were found in the *pilI* and *chpA* genes, respectively, whereas no other CheW-like homologues—such as the mentioned orthologue of *P. aeruginosa chpC*—were found in the *R. solanacearum* GMI1000 genome. Compared to its *P. aeruginosa* counterpart (PAO1 protein WP_003084590), the *R. solanacearum* PilI homologue presents an identity level of 68%, with fragment coverage of 31%, whereas the *R. solanacearum* ChpA homologue shows an identity level of 39.73% with 33.33% coverage with respect to the *P. aeruginosa* ChpA (WP_003114893) protein. To determine whether the five *pil-chp* genes are part of the same transcriptional unit, reverse transcription-PCRs (RT-PCRs) were performed (Fig. 1C). The resulting bands confirmed that the *pil-chp* gene cluster is transcribed as a single polycistronic unit, transcriptionally independent of the surrounding *RSc0667* and *RSc0673* genes (Fig. 1C), predicted to encode a rubredoxin protein and a hypothetical protein, respectively.

The *R. solanacearum* PilI and ChpA proteins are involved in twitching but not in swimming motility or chemotaxis. To determine the role of *pilI* and *chpA* in *R. solanacearum* motility and chemotaxis, we created null mutants by replacing their protein-coding sequences with a kanamycin cassette. Strains with an inactivated *pilA*, *fliC*, or *cheA* gene were also constructed to be used as controls: the *pilA* mutant was described previously as impaired in twitching (8), the *fliC* mutant as deficient in swimming (5), and the *cheA* mutant as nonchemotactic (6). All mutants obtained were confirmed by PCR (see Fig. S1 in the supplemental material) and subsequent sequencing (Macrogen) using specific primers (see Table S1 in the supplemental material). Furthermore, none of the constructed knockout mutants exhibited macroscopic changes in colonial shape, EPS production (Fig. S2), or growth rate *in vitro* (Fig. S3).

After growth in the appropriate solid medium, colonies of the wild-type (WT) *R. solanacearum* GMI1000 strain exhibited a normal twitching phenotype characterized by irregular colony edges with multiple projections easily observed under light microscopy (Fig. 2A). In contrast, the *pilI* mutant presented round-shaped colony margins without projections, a phenotype identical to that of the nontwitching motility control *pilA* mutant (Fig. 2A). The *chpA* mutant strain also showed impaired twitching movement, but unlike the *pilI* and *pilA* mutants, the reduced twitching motility in the *chpA* mutant was characterized by smaller projections in the colony margins, indicating some residual movement (Fig. 2A). As expected, the *fliC* flagellum mutant control strain presented a twitching phenotype similar to that of the WT GMI1000 strain (Fig. 2A). It is worth noting that the complementation of the *pilI* mutant restored twitching motility, discarding polar effects on downstream genes caused by the *pilI* disruption or by secondary mutations (Fig. S4A).

It was reported previously that inactivation of TFP genes might modify the motility controlled by flagella and vice versa (12–14). Thus, we analyzed the swimming capacity in *pilI* and *chpA* mutants, including again as controls the *pilA* and the *fliC* knockouts, known to be affected only in twitching and swimming motility, respectively (5, 8). After growth in the appropriate semisolid medium, the *pilI* and *chpA* mutants exhibited a typical swimming halo around the inoculated area, similar to that of the WT strain, whereas the *fliC* control mutant was completely impaired in this type of motility, as expected (Fig. 2B). Surprisingly, the *pilA* mutant strain displayed an increased swimming halo compared to that of the WT parental strain (Fig. 2B). Bacterial swimming was more accurately quantified by measuring the dispersion halo at 72 h (Fig. 2C). Significant ($P < 0.05$) differences—more remarkable over time—between the *pilA* mutant and the rest of strains (the WT strain and the *pilI* and *chpA* mutants) showing a normal swimming phenotype were recorded (Fig. 2C). As previously described, the *fliC* control strain showed a significantly reduced swimming halo (Fig. 2C).

In order to determine whether these motility patterns affected bacterial chemotaxis, capillarity assays were performed using Casamino Acids as a chemoattractant (6). Since

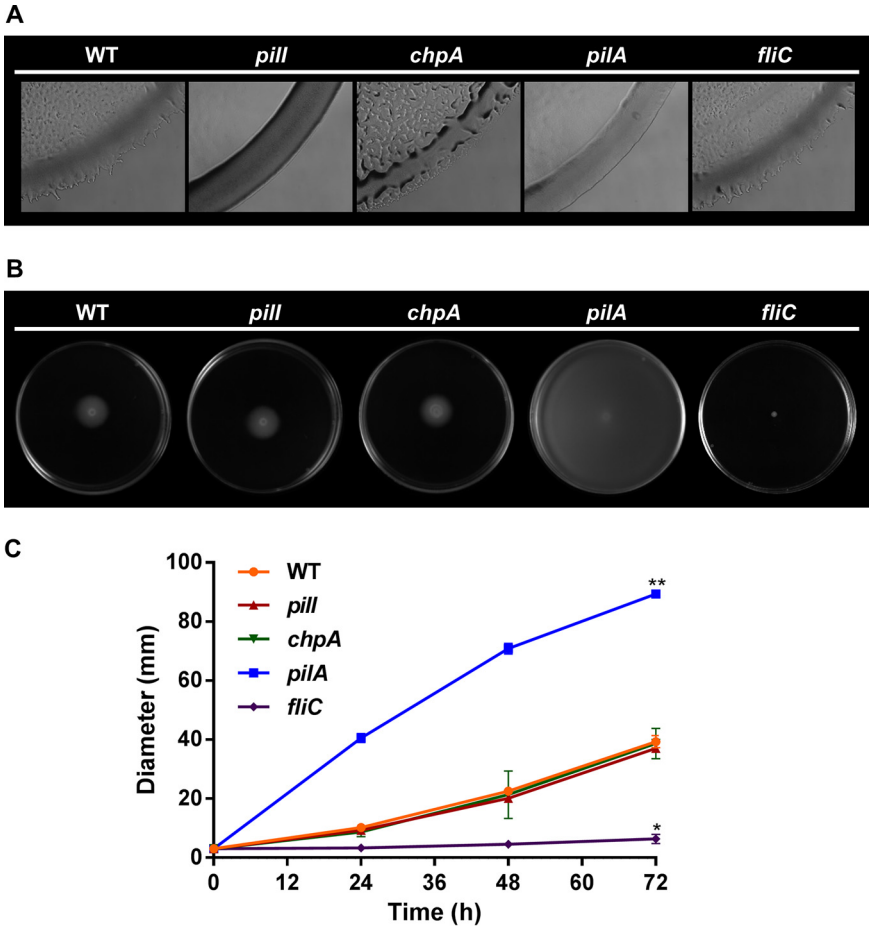


FIG 2 Motility assays. (A) Representative optical microscope images (×100 magnification) of three independent twitching motility assays. (B) Representative images of three independent swimming motility assays. (C) Representation of the swimming halo diameters measured in three independent assays with three replicates each. Error bars represent standard deviations of the means, and significant ($P < 0.05$) differences from the *R. solanacearum* WT strain are represented as single or double asterisks for a bacterial halo of smaller or larger diameter, respectively.

the *fliC* mutant lacked swimming motility, a *cheA* mutant strain—whose chemotactic response was abolished (6)—was constructed and included in the assays as a more appropriate control. In the parental strain and TFP-related mutants, an approximately 20-fold increase in bacterial counts was observed in capillaries filled with Casamino Acids relative to those containing only chemotaxis buffer (Fig. 3). Thus, with the exception of the motile but nonchemotactic *cheA* knockout, no significant differences in chemotaxis were observed in any TFP-related knockout compared to the WT strain (Fig. 3).

The *R. solanacearum pilI* and *chpA* mutants present reduced natural transformation abilities. The TFP are essential for bacteria to carry out natural transformation (8). Thus, in order to examine their natural transformation abilities, the WT strain and the corresponding *pilI*, *chpA*, *pilA*, and *fliC* knockout mutant counterparts were exposed to DNA containing a gentamicin cassette flanked by ~1-kb-long sequences homologous to a noncoding region of the *R. solanacearum* genome (15) and the frequencies of recovery of gentamicin-resistant colonies were calculated. The results obtained showed that the transformation frequencies of the *pilI* and *chpA* mutants were reduced by approximately 20-fold and 6-fold, respectively, compared to the WT strain (Table 1). As expected, the *pilA* mutant, which lacks TFP, was totally unable to take up DNA naturally (8), whereas the *fliC* mutant was transformed with a level of efficiency comparable to that of the WT strain (Table 1).

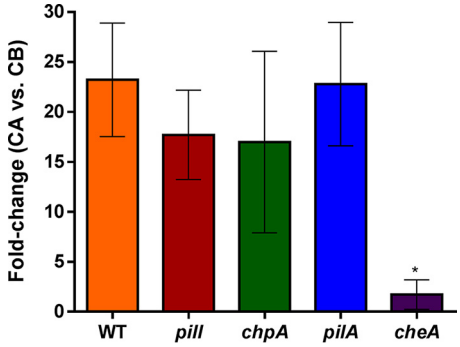


FIG 3 Chemotaxis capillarity assays. Data represent fold change (CFU) between viable bacteria counted in capillaries containing chemoattractant CA (Casamino Acids) divided by the CFU counted in control capillary CB (chemotaxis buffer). Error bars represent standard deviations of the means of results from five replicates per strain, and the asterisk denotes a significant ($P < 0.05$) difference from the *R. solanacearum* WT strain. The assay was performed three times. The results of a representative experiment are shown.

TFP- and flagellin-associated genes are involved in biofilm formation and attachment of *R. solanacearum* to plant roots. Alongside roles in motility, TFP and flagella are required for biofilm formation and initial bacterial adsorption to plant roots (16). We thus measured the capacity of our bacterial mutants to produce biofilm in polystyrene microplate cultures. The *pilA* control mutant was previously shown to produce less-developed biofilms than a WT strain (8), which was quantified here as a significant ($P < 0.05$) reduction (~70%) in biofilm formation. The *chpA* and *fliC* mutants also displayed comparable ~70% reductions in their ability to produce biofilm compared to the GMI1000 strain (Fig. 4A). Interestingly, the *pilI* mutant, which exhibited an abolishment of twitching motility similar to that shown by the nonmotile *pilA* mutant, presented a significant ($P < 0.05$) increase of ~25% in biofilm formation with respect to the WT parental strain (Fig. 4A). Furthermore, the complemented *pilI* mutant showed a restored ability to produce biofilm (Fig. S4B).

Next, we incubated each bacterial strain with isolated tomato roots and quantified their capacity to attach to the root surface. The results of these experiments showed that all TFP mutants (*pilI*, *chpA*, and *pilA*) presented a statistically significant ($P < 0.05$) 10-fold-lower level of root attachment than the WT GMI1000 strain (Fig. 4B). Furthermore, the aflagellated *fliC* mutant also displayed a statistically significant ($P < 0.05$) 5-fold decrease compared with the WT strain (Fig. 4B), denoting that both TFP and flagella promote adhesion between *R. solanacearum* cells and tomato roots.

The *pilI* and *chpA* mutants show reduced virulence in soil-soak inoculations but not when directly inoculated in the stem of tomato plants. To determine the effect of PilI and ChpA on *R. solanacearum* pathogenicity, tomato plants were infected by drenching the soil with the collected bacterial solution without wounding the roots, which mimics the natural infection process of this soilborne pathogen. The four mutants analyzed (*pilI*, *chpA*, *pilA*, and *fliC*) exhibited a significant ($P < 0.05$) reduction in their ability to develop plant wilting compared to the parental WT strain, but to

TABLE 1 Natural transformation frequencies of the indicated *R. solanacearum* strains[a]

Strain	Natural transformation frequency[b]
GMI1000 WT	$1.05\ (\pm 1.2) \times 10^{-6}$
pilI	$5.07\ (\pm 4.9) \times 10^{-8}$
chpA	$1.78\ (\pm 1.5) \times 10^{-7}$
pilA	$<2.57 \times 10^{-9}$
fliC	$1.66\ (\pm 2.1) \times 10^{-6}$

[a]Each experiment was carried out in triplicate in five independent assays.
[b]Natural transformation frequency data are represented as means ± standard deviations and were calculated as the number of recombinant colonies by the total number of viable cells. At least 10% of the recombinant colonies obtained for each strain were confirmed by sequencing.

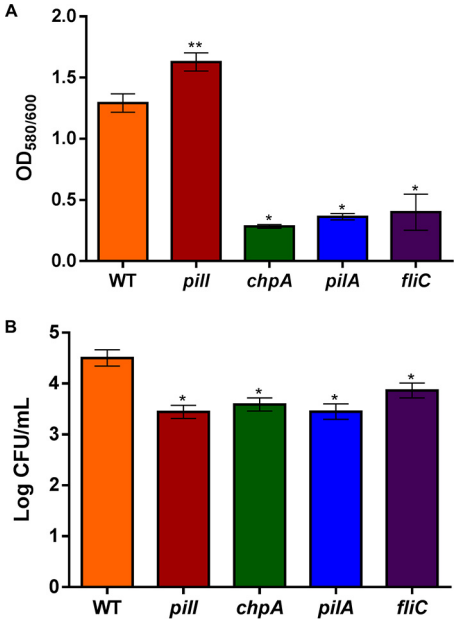


FIG 4 Biofilm and root attachment quantification. (A) Biofilm assay in which *y*-axis data represent biofilm absorbance (OD_{580}) divided by the biomass (OD_{600}). The error bars represent standard deviations of the means of results from 16 replicates per strain. Significant ($P < 0.05$) differences from the *R. solanacearum* WT strain are represented as single or double asterisks for lower or higher absorbance ratio values, respectively. The assay was performed three times. The results of a representative experiment are shown. (B) Representative root attachment assay showing means of logarithms of counts of viable bacteria (CFU). Error bars represent standard errors of the means of data from five 1-week-old tomato roots per assay, and asterisks denote significant ($P < 0.05$) differences from the *R. solanacearum* WT strain. The assay was performed three times. The results of a representative experiment are shown.

differing degrees (Fig. 5A). Statistical analysis classified the mutants into four groups: the *pilA* deletion mutant was the least virulent, followed by the *pilI* and *chpA* mutants, with both showing an intermediate phenotype, and the *fliC* mutant, with a small but significant decrease in apparent wilting compared to the WT parental strain (Fig. 5A). Bacterial counts obtained from 3-cm stem cuts from tomato plants infected using the same procedure were also carried out at 4, 8, and 12 dpi (days postinoculation). Only the *pilA* mutant exhibited a significant (2 log) reduction at days 4 and 12 ($P < 0.05$) with respect to the rest of strains, whose numbers in stem tissues were similar to those seen with the WT strain (Fig. 5B). To discard any potential fitness effects resulting from the growth of the knockout strains in tomato plants, all strains were infiltrated in tomato leaves. The results showed no differences in bacterial growth of any of the mutants with respect to the WT (Fig. S5). In contrast, when tomato plants were infected by direct petiole injection, *pilI* and *chpA* knockout strains showed no statistically significant reduction in their capacity to wilt plants (Fig. 6A), while the control *pilA* mutant exhibited significant ($P < 0.05$) differences in disease index in comparison to the WT, as previously described (8). Finally, the virulence of the flagellum-deficient *fliC* mutant was also comparable to that of the WT strain (Fig. 6A). Bacterial counts measured over time in infected plant stems were similar to those reached by the WT strain for all mutants except for the *pilA* mutant, which presented a significant ($P < 0.05$) reduction in plant colonization (Fig. 6B). Interestingly, the *fliC* mutant exhibited a reduction in stem numbers only at 3 dpi, reaching values similar to those shown in the *pilI* and *chpA* mutants and the WT strains at later infection times (Fig. 6B).

Deletion of *pilA* but not *pilI*, *chpA*, and *fliC* limits bacterial spread in plant tissues. In order to study the distribution of *pilI*, *chpA*, *pilA*, and *fliC R. solanacearum* knockout mutants along tomato plants, reporter strains were constructed through the insertion into their genome of the *luxCDABE* operon under the control of the *hrpB* promoter (Fig. S6). Tomato plants grown in pots were soil-inoculated with the reporter

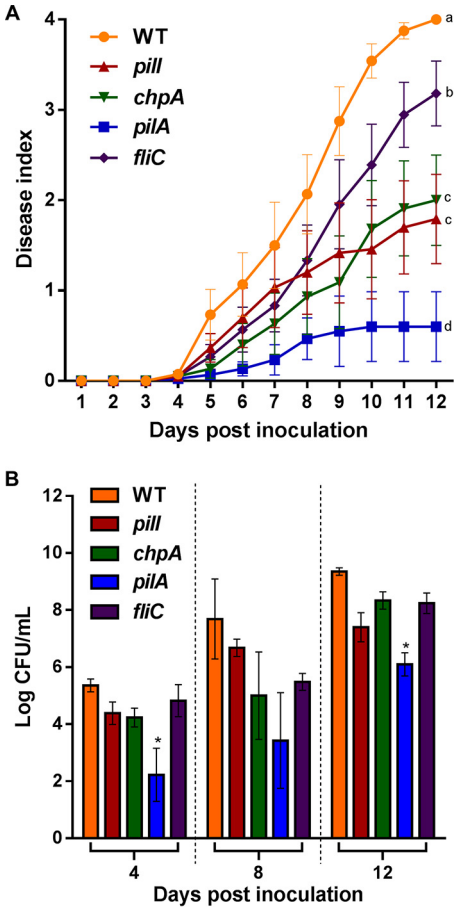


FIG 5 Drenching assays. (A) Disease index scaled from 0 (no wilt) to 4 (death), with levels measured daily after soil soaking of 4-week-old tomato plants by the use of a naturalistic inoculation method. Error bars represent standard errors of the means of results from 20 replicates per strain. According to their wilting reduction ($P < 0.05$), strains are classified in four groups (labeled a through d). The assay was performed three times. The results of a representative experiment are shown. (B) Logarithm of counts of viable bacteria (CFU per milliliter) after soil soaking of 4-week-old tomato plants by the use of a naturalistic soil soak inoculation method at 4, 8, and 12 days postinoculation. Error bars represent standard errors of the means of results from 20 replicates per strain. Asterisks denote significant ($P < 0.05$) differences from the *R. solanacearum* WT strain. The assay was performed three times. The results of a representative experiment are shown.

strains, and luminescence in different stem sections was recorded at 3 and 6 dpi. At 3 dpi, no significant differences were observed between any of the knockouts and the WT strain at any stem height (Fig. 7). However, at 6 dpi, the *pilA* mutant carrying the *luxCDABE* operon exhibited a significant ($P < 0.05$) reduction of its luminescence compared to the WT reporter strain (Fig. 7). No significant differences in colonization between the stem sections from internodes 2 and 3 were observed for any other strain at 6 dpi (Fig. 7). To confirm that expression of the reporter operon was not affected by any of the gene disruptions, bacterial growth and luminescence were both measured over time in *in vitro* cultures (Fig. S7). In these experiments, all mutants showed comparable levels of luminescence and indistinguishable differences in growth from the WT strain, ruling out an inhibition of the reporter in the *PilA* mutant (Fig. S7).

DISCUSSION

A conserved cluster involved in twitching motility. In this study, we identified a new gene cluster in *R. solanacearum* strain GMI1000 (Fig. 1B) presenting synteny with respect to *P. aeruginosa* and *Lysobacter enzymogenes* cluster IV (17–19) and the *Xylella fastidiosa pil-chp* operon (20). With the construction of pilus- and ChpA-deficient strains, we demonstrated the involvement of these genes in *R. solanacearum* twitching but not

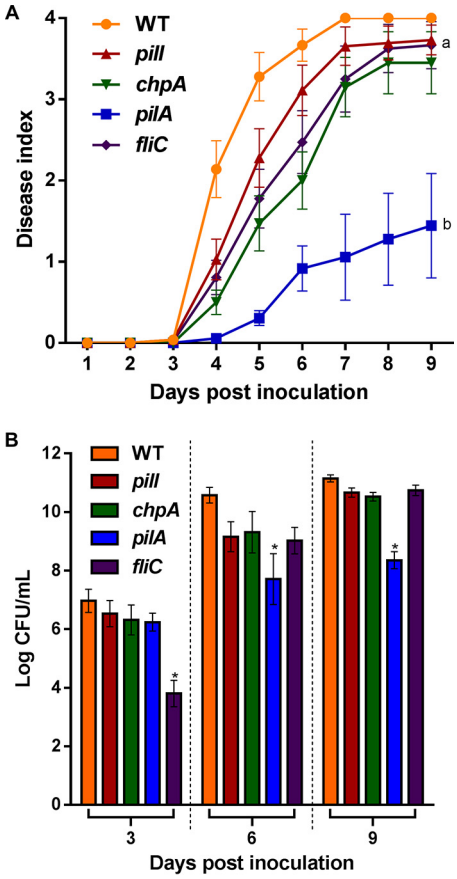


FIG 6 Petiole inoculation assays. (A) Disease index scaled from 0 (no wilt) to 4 (death), measured daily in 4-week-old tomato plants after the use of a direct inoculation method. Error bars represent standard errors of the means of results from 20 replicates per strain. According to statistically significant differences ($P < 0.05$), strains were classified in two groups (labeled a and b). The assay was performed three times. The results of a representative experiment are shown. (B) Logarithms of viable bacteria (CFU per milliliter) of the indicated *R. solanacearum* strains counted by the use of a direct inoculation method using 4-week-old tomato plants at 3, 6, and 9 dpi. Error bars represent standard errors of the means of results from 20 replicates per strain, and asterisks denote significant ($P < 0.05$) differences from the *R. solanacearum* WT strain. The assay was performed three times. The results of a representative experiment are shown.

in swimming (Fig. 2). Whereas the *chpA* mutant presented reduced twitching, PilI inactivation produced a total abolition of this movement, comparable to that seen with the nontwitching *pilA* control (Fig. 2A). Decreased twitching motility has been similarly observed in *chpA* (CheA-like) knockout mutants in other bacteria such as *P. aeruginosa* (21) and *X. fastidiosa* (20) and in the *pilI* (CheW-like) null mutant of *P. aeruginosa* (22). However, the *pilI* knockout remains twitching proficient in *L. enzymogenes* (23), indicating differences in TFP gene function that depend on the bacterial species. In contrast to our results seen in *R. solanacearum* revealing that the *chpA* mutant showed some residual twitching motility, both *pilI* and *chpA/pilL* mutants in *Acidovorax citrulli* lacked twitching motility and did not produce TFP (24). However, a *pilJ* mutant in the *A. citrulli pil-chp* operon retained the ability to produce TFP (13).

TFP influence swimming motility. Since the inactivation of specific genes associated with one type of appendage may affect the movement controlled by others (12–14, 25), swimming motility assays were also performed with the *R. solanacearum* mutants that had been constructed. Surprisingly, results obtained showed swimming hypermotility performed by the *pilA* mutant (Fig. 2B and C), a phenomenon observed in other *R. solanacearum* knockouts such as those lacking the transcriptional regulators *phcA* (26) and *motN* (27), the latter being chemotaxis proficient like the *R. solanacearum*

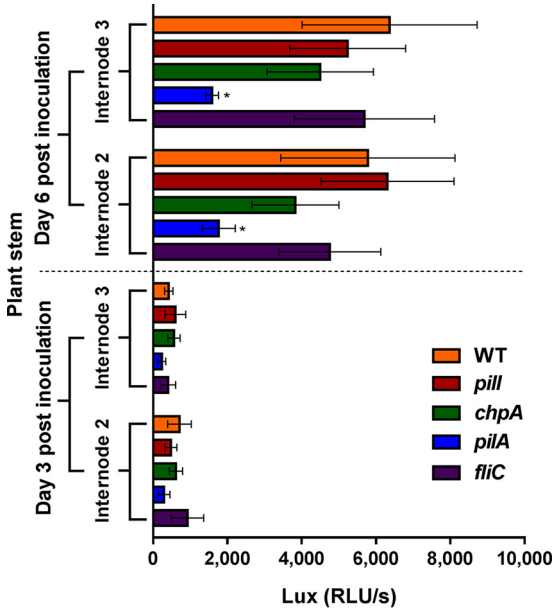


FIG 7 Bacterial spread in plant tissue. Luminescence detection of the indicated *R. solanacearum* strains prepared by the use of a direct inoculation method was performed using 4-week-old tomato plants at 3 and 6 dpi. *x*-axis data represent means of luminescence (RLU/s) data from 10 replicates per strain. Error bars represent standard errors of the means, and asterisks denote significant ($P < 0.05$) differences from the *R. solanacearum* WT strain. The assay was performed three times. The results of a representative experiment are shown.

pilA mutant (Fig. 3). Cross-effects between the two appendage-dependent movements are likely underestimated due to the comparative paucity of studies in which both swimming and twitching assays have been carried out to evaluate the effect of single mutants. One example of this connection is found in the PilS-PilR two-component system of *P. aeruginosa*, which regulates TFP expression and whose inactivation also causes a reduction in swimming motility (25). Similarly, the inactivation of genes encoding PilA, the pilus assembly protein PilO, or two predicted minor pilins, FimU and FimT, caused reduced twitching but complete abolition—or, in the case of the *fimT* mutant, impairment—of swimming in *P. syringae*, suggesting that TFP are involved not only in twitching but also in swimming (14).

***R. solanacearum* Pill and ChpA play a role in all known TFP-related functions.** As previously reported in *R. solanacearum* studies of the *pilA* mutant, TFP are required for natural transformation and biofilm formation (8). Our results demonstrate that Pill and ChpA also contribute to natural competence (Table 1), presumably through proper regulation of TFP. Regarding biofilm formation, our data showed that Pill and ChpA play contrary roles (Fig. 4A). Interestingly, knockouts lacking either PilA or PilQ in *Xanthomonas* spp. displayed reduced twitching motility, but biofilm formation was affected only in the *pilQ* mutant (28, 29). On the other hand, in the *A. citrulli fliR*-null mutant, whose swimming and twitching movements remained impaired, no effect on biofilm development was observed (12). Besides their roles in natural transformation and biofilm formation, TFP are also key for bacterial virulence (7). In *R. solanacearum*, virulence processes during plant colonization have been investigated in some TFP-related genes such as *pilA* (8) and *pilQ* (30), highlighting the relationship between twitching motility and virulence (31–33). Our data demonstrate that Pill and ChpA proteins are required for early pathogenic stages that result in effective plant colonization and wilting. This is shown by the fact that strains deleted of these genes caused reduced wilting in response to a naturalistic infection method (soil drenching; Fig. 5A) but behaved like the WT when applied directly by petiole inoculation, a procedure that overcomes all initial steps of plant colonization until the bacterium reaches the xylem (Fig. 6A). Similarly, our data corroborate the idea that PilA plays a role in the pathogenesis of *R.*

solanacearum, as previously reported (5, 8), because this mutant showed impaired multiplication *in planta* in both root drenching and petiole inoculation experiments (Fig. 5B and 6B) and restricted stem colonization (Fig. 7). Involvement of TFP gene inactivation and virulence has also been described in several bacterial plant pathogens such as *P. syringae* (14), *Xanthomonas* spp. (28, 34), *A. citrulli* (13), and *X. fastidiosa* (20, 35), in which an impairment of twitching motility resulted in the development of a reduced pathogenic phenotype. Remarkably, although all TFP mutants analyzed showed similar phenotypes in biofilm formation, root attachment, and chemotaxis (Fig. 3 and 4), the *pilI* and *chpA* deletion mutants showed milder phenotypes than the *pilA* mutant in transformation efficiency and in the various virulence and plant colonization assays performed (Table 1; see also Fig. 5 and 7). This can be explained by the fact that, while PilA is the structural pilus subunit and its mutant is nonpilliated, the PilI and ChpA are regulator proteins in TFP assembly and their deletion mutants may still present some TFP, as indicated by the residual twitching motility displayed by the *chpA* knockout strain (Fig. 2A). In this sense, it is worth mentioning that disruption of the *chpA* homolog gene in *X. fastidiosa* (*pilL*) resulted in a loss of twitching motility but in retention of the ability to produce TFP, resembling the phenotype of the *R. solanacearum chpA* mutant, although in the latter some residual twitching motility was observed, maybe because of differences in the experimental settings.

Novel roles of flagella in *R. solanacearum* GMI1000. This work demonstrates that adhesion to roots via TFP is crucial for optimal plant colonization and disease development but also that flagella are involved in these processes (Fig. 4B). Implication of flagella in attachment to both animal and plant cells has been recently reported in bacterial pathogens at the early stage of infection (36–38). Although flagella have not been reported to play a role in attachment of members of the *Xanthomonadaceae* (39), in other bacterial species such as *Azospirillum brasilense*, flagellin-deficient mutants are impaired in attachment to wheat roots, and the purified polar flagellum binds directly to the wheat root surface (40).

In addition, our results shown that *fliC* inactivation produces a reduction in biofilm formation (Fig. 4A), indicating that flagella also contribute to this process in *R. solanacearum* GMI1000, as had been observed in *P. aeruginosa* and other bacteria (41, 42). In contrast, inactivation of either *fliC* or genes involved in aerotaxis—an active cell movement along oxygen gradients—in *R. solanacearum* strain K60 caused increased biofilm production (43), indicating strain-specific TFP functions.

The flagellar protein FliC was previously shown to play a role in pathogenesis of *R. solanacearum*, especially during the first steps of the interaction (5, 8). Here, we also observed that the *fliC* deletion mutant caused reduced wilting only when inoculated by root drenching and not by direct petiole inoculation (Fig. 5A and 6A). However, this flagellum-deficient mutant was affected only slightly in its capacity to multiply *in planta* (Fig. 6B) and colonize the plant stem (Fig. 7), in contrast with the stronger phenotypes shown by the nonpilliated *pilA* strain. We conclude that TFP are more important than flagella for the interaction of *R. solanacearum* with tomato plants.

Conclusion. In this work, we have demonstrated that the virulence of *R. solanacearum pilI*, *chpA*, *pilA*, and *fliC* deletion mutants is impaired in the first stages of plant colonization and that the *pilA* mutant shows decreased growth after drenching or petiole inoculation. This is the first report on the putative *R. solanacearum* type IV pilus regulators PilI and ChpA, where we clearly demonstrate their role in twitching motility, biofilm formation, natural transformation, and virulence. Additionally, a hypermotile swimming phenotype in GMI1000 strain lacking PilA and the role of FliC in root attachment and biofilm formation have been described here for the first time. Our work suggests that further research in *R. solanacearum* should be addressed to elucidate putative connections between swimming and twitching motilities in both well-documented genes and genes with unknown function.

TABLE 2 Bacterial strains and plasmids used in this work[a]

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	*E. coli supE4* Δ*lacU169* (*80* Δ*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Clontech
R. solanacearum GMI1000	Wild-type strain (phylotype I, race 1 biovar 3)	45
R. solanacearum GMI1000 PhB-lux	GMI1000 strain with *PhrpB-lux* from pRCGent-PhB-lux, Gm^r	This work
R. solanacearum pilI	GMI1000 strain with Δ*pilI*::*loxP-Km* from pCM184, Km^r	This work
R. solanacearum pilI PhB-lux	PilI with *PhrpB-lux* from pRCGent-PhB-lux, Km^r, Gm^r	This work
R. solanacearum chpA	GMI1000 strain with Δ*chpA*::*loxP-Km* from pCM184, Km^r	This work
R. solanacearum chpA PhB-lux	ChpA strain with *PhrpB-lux* from pRCGent-PhB-lux, Km^r, Gm^r	This work
R. solanacearum pilA	GMI1000 strain with Δ*pilA*::*loxP-Km* from pCM184, Km^r	This work
R. solanacearum pilA PhB-lux	PilA strain with *PhrpB-lux* from pRCGent-PhB-lux, Km^r, Gm^r	This work
R. solanacearum fliC	GMI1000 strain with Δ*fliC*::*loxP-Km* from pCM184, Km^r	This work
R. solanacearum fliC PhB-lux	FliC strain with *PhrpB-lux* from pRCGent-PhB-lux, Km^r, Gm^r	This work
R. solanacearum cheA	GMI1000 strain with Δ*cheA*::*loxP-Km* from pCM184, Km^r	This work
Plasmids		
pCM184	Allelic exchange vector, carrying kanamycin cassette flanked by *loxP*, Ap^r, Km^r	Addgene
pGEMT	Cloning vector, Ap^r	Promega
pRCGent-Pps-GWY	Vector carrying gentamicin cassette flanked by regions homologous to the GMI1000 genome, Ap^r, Gm^r	15
pRCGent-PhB-lux	Vector carrying *luxCDABE* operon from pMU1 cloned in KpnI–NotI in pRCGent-PhB, Ap^r, Gm^r	49
pBT4	Broad-host-range vector carrying the pBBR1 replicon, Tc^r	Addgene
pDSK-GFPuv	Vector carrying the *PpsbA* promoter, Km^r	LiveScience

[a]Km^r, Gm^r, Ap^r, and Tc^r stand for resistance to kanamycin, gentamicin, ampicillin, and tetracycline resistance, respectively.

MATERIALS AND METHODS

Bacterial strains, plasmids, plant material, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* DH5α was grown at 37°C in Luria-Bertani (LB) (44) agar or in LB broth with shaking at 180 rpm. The *R. solanacearum* GMI1000 WT strain and derivate mutants were routinely grown at 28°C in rich B medium, Boucher's minimal medium (MM) (45), and CPG (Casamino Acids-peptone-glucose) medium (46) agar or broth with shaking at 180 rpm. When necessary, rich B medium was supplemented with 0.5% glucose and 0.005% 2,3,5-triphenyltetrazolium chloride (final concentration) in agar plates, and MM broth was supplemented with 2% glycerol or 20 mM glutamate (final concentration). When needed, ampicillin (50 mg/liter), kanamycin (50 mg/liter), gentamicin (10 mg/liter), or tetracycline (5 mg/liter) was added in growth media. For phytopathogenesis assays, tomato plants (*Solanum lycopersicum* cultivar Marmande) were used to evaluate virulence of *R. solanacearum*. Plants were routinely grown in pots in a mixed soil of Substrate 2 (Klasmann-Deilmann GmbH, Geeste, Germany), perlite, and vermiculite at a proportion of 30:1:1 for 1 to 4 weeks at 22°C and 60% relative humidity under long-day light regimen conditions (16 h light and 8 h darkness). Before the infectious assays, tomato plants were acclimated at least 3 days by transferring them to a growth chamber at 27°C under the same humidity and photoperiod conditions.

RNA extraction and RT-PCR. Total RNA was extracted by the use of an RNeasy minikit (Qiagen, Hilden, Germany) from 5-ml cultures at an absorbance level of 0.4 (optical density at 600 nm [OD_{600}]) that had previously been pelleted and treated with lysozyme (50 mg/ml) and resuspended in Tris-EDTA (TE) buffer for 10 min at 37°C. Once extracted, the RNA was incubated with DNase Turbo Ambion (Thermo Fisher, Waltham, MA) to remove DNA contaminants, and PCR analysis of the mixtures performed using RNA samples without reverse transcriptase confirmed the absence. Reverse transcription of RNA was performed through the use of a first-strand cDNA synthesis kit (Nzytech, Lisbon, Portugal) and the appropriate primers (listed in Table S1 in the supplemental material). All molecular techniques were performed using standard procedures.

Gene identification and construction of *R. solanacearum* knockouts and of complemented and reporter strains. The *P. aeruginosa* PilI and ChpA homologues in *R. solanacearum* were identified through mapping coded proteins to nonsupervised orthologous groups (NOGs) using the eggNOG v4.0 Web service (47) and complete *R. solanacearum* GMI1000 chromosome (NC_003295.1) and megaplasmid (NC_003296.1) sequences. Knockout deletion mutants *pilI* (WP_011000625), *chpA* (WP_011000627), *pilA* (WP_011000517), *fliC* (WP_011003694), and *cheA* (WP_011004658) were generated by replacing the coding sequence of each target gene by a kanamycin resistance cassette as described previously (48). To this end, DNA fragments corresponding to ~1 kb of the upstream and downstream flanking regions of each gene and a kanamycin cassette flanked with *loxP* regions were PCR amplified from the *R. solanacearum* GMI1000 genome and plasmid pCM184 (Addgene), respectively. The oligonucleotides used (Table S1) included overlapping regions enabling the three fragments to be fused in two amplification rounds. All PCRs were performed using proofreading Phusion High-Fidelity DNA polymerase (Thermo Fisher). Next, 3′ A overhangs were added to the final PCR products using *Taq* polymerase (Invitrogen, Waltham, MA) and the fragments were cloned into pGEM-T plasmid (Promega, Madison, Wisconsin), transformed in *E. coli* DH5α, and selected in ampicillin- and kanamycin-containing LB plates.

Sequenced constructs (Macrogen, Seoul, South Korea) were amplified with appropriate primers (Table S1), and the final product was purified (NZYGelpure; Nzytech), naturally transformed in *R. solanacearum* strain GMI1000, and plated in kanamycin B rich medium for mutant selection (49).For complementation, the *PpsbA* promoter and the corresponding gene were amplified from the pDSK-GFPuv (LifeScience) plasmid and the *R. solanacearum* GMI1000 genome, respectively. Then, both fragments were cloned into the pBT4 vector (Addgene) using Gibson assembly master mix (New Engand Biolabs, Ipswich, Suffolk, United Kingdom) and the appropriate primers (Table S1). Knockouts were then electroporated with the constructed plasmid and selected in tetracycline-containing medium plates. To construct luminescent reporter strains, integration of the *luxCDABE* operon under the *hrpB* promoter was carried out as previously described (49). Briefly, the reported operon was amplified from the pRCGent-PhB-lux plasmid using the appropriate primers and electroporated to each strain. Transformants were selected on gentamicin-containing B rich plates. All knockouts and reported strain verifications were carried out by PCR amplification and sequencing (Macrogen) with the corresponding primers indicated in Table S1.

Twitching and swimming assays. Modified CPG plates with 0.3% and 1.6% Difco agar were used on the day of their preparation for analysis of swimming and twitching motilities, respectively (50). Both motility tests were carried out by the inoculation of a 2-μl drop of a bacterial suspension into Mill-Q (MQ) water at an absorbance level of 0.1 (OD_{600}) in the middle of the appropriate plate. After inoculation, both CPG-type plates were incubated at 30°C and 24 h for twitching (until layered edges with multiple irregular projections were observed in the WT strain) and 72 h for swimming (measuring the bacterial halo daily). A Zeiss AxioImager M2 microscope (Carl Zeiss Microscopy, Oberkochen, Germany) was used to obtain representative images of twitching motility at 40× increases. A ChemiDoc XRS+ system (Bio-Rad, Hercules, CA) was used to obtain swimming motility images.

Chemotaxis capillary assays. To establish chemotactic effects in *R. solanacearum* knockouts, capillary assays were carried out as described previously (51), with some modifications. Briefly, three V-shaped bent needles (Nipro; Kita-ku, Osaka, Japan) covered with a 24-by-65 mm microscope coverslip (Menzel-Glässer; VWR, Radnor, PA) were placed on the surface of aseptic 140-mm-diameter petri dishes (Deltalab; Rubí, Barcelona, Spain) to form the chemotaxis chambers. Sealed and autoclaved 1-ml capillaries (Microcaps, Drummond Scientific Co., Broomall, PA) (3 cm in length) were filled with chemotactic buffer (CB) as a negative control or with CB with 2% Casamino Acids as a chemoattractant (6). Bacterial suspensions were prepared from overnight cultures in B rich medium with the appropriate antibiotics, washed twice with MQ water, and adjusted to an OD_{600} of 0.1. Chemotaxis chambers were filled with the corresponding bacterial suspensions and incubated at 30°C during 2 h. Once incubated, the capillaries were washed twice to remove any external attached cell and then broken off, and the content was poured into a microcentrifuge tube containing 1 ml of MQ water. Proper dilutions were plated in rich B medium, and the CFU counts per milliliter obtained under each set of conditions were normalized between the capillaries treated with 2% Casamino Acids and those left untreated.

Natural transformation assays. Natural transformation efficiencies were determined as previously described (52). Briefly, 100 ng of DNA containing a gentamicin cassette with homologous regions amplified from pRCG-Pps-GWY (53) was added to each 50-μl volume of the corresponding strains grown in MM broth supplemented with 2% glycerol. Transformed clones were selected on gentamicin-containing B rich medium plates and verified by PCR and sequencing (Macrogen) with the corresponding primers indicated in Table S1. The same cultures were also plated in B rich medium without antibiotic to obtain the total number of viable bacteria (CFU per milliliter). Transformation frequencies were calculated as the number of recombinant colonies divided by the total number of CFU per milliliter.

Biofilm quantification. Quantitative analysis of *R. solanacearum* biofilm formation was carried out through crystal violet assay by the use of a method adapted from previous work (54). Briefly, CPG overnight cultures were collected, washed, and adjusted in CPG to an OD_{600} of 0.1. Next, 95 μl of CPG broth and 5 μl of each bacterial suspension were added onto 96-well polystyrene microplates (Greiner, Kremsmünster, Austria) and incubated without shaking at 30°C during 24 h. After incubation, biomass growth in wells was measured at an OD_{600}. Subsequently, wells were washed twice with MQ water, incubated with 100 μl of 0.1% crystal violet stain, and incubated at room temperature for 30 min. Wells were then washed with MQ water three times, and the stained biofilm immobilized on the wells was solubilized with 100 μl of 95% ethanol and measured at OD_{580}. Measurements were performed using a multiplate reader (Sunrise, Tecan, Zürich, Switzerland), and results were normalized according to previously obtained biomass absorbance (OD_{580}/OD_{600}).

Root attachment quantification. Attachment to tomato roots was carried out as previously described (55), with slight modifications. One- or 2-week-old plants, grown as described above without previous acclimation, were collected and washed. Roots were submerged in 1 ml of bacterial culture (approximately 10^6 CFU), obtained from a diluted culture at an OD_{600} of 0.1 (~10^8 CFU/ml) in MQ water. Submerged root plants were incubated at 25°C with gentle shaking at 50 rpm during 4 h to promote bacterium-root contact. After incubation, roots were placed in tubes with 30 ml sterile 0.88% NaCl, shaken during 1 min at 200 rpm to remove unattached bacteria, and gently dried to remove liquid excess. Attached bacteria were then collected by immersing 2-cm-long dried roots in tubes with 1 ml sterile 0.88% NaCl, followed by 1 min of vortex mixing. Appropriated dilutions were plated in B rich medium.

Bacterial leaf multiplication assays. Bacterial growth *in planta* was measured as previously described (56), with some modifications. A 3-liter inoculum of each bacterial strain was prepared, starting from a culture maintained at an OD_{600} of 0.1 in MQ water (approximately 10^8 CFU/ml) to obtain a final concentration of ~10^5 CFU/ml. Infections were performed using a vacuum machine, and to reduce the

surface tension of the water, 150 μl of Silwet L-77 was added to the 3-liter inoculum. Four-week-old tomato plants were submerged in the respective bacterial inocula, and vacuum was applied during 1 min to promote bacterial infiltration in leaves. After inoculation, plants were retrieved, dried, and incubated under standard conditions (27°C, 60% relative humidity, and 16-h light/8-h darkness). At specific dpi, four 5-mm^2 leaf disks were recovered from the infiltrated plants, collected in tubes containing 200 μl of MQ water, and subjected to a grinding process. The collected dilutions were plated on B medium to obtain the final concentration of each strain on leaf surface (expressed as CFU count per square centimeter).

Bacterial virulence experiments. For drenching assays, 4-week-old unwounded plants were soil-soak inoculated by watering with 40 ml of bacterial culture, at an OD_{600} of 0.1 (~10^8 CFU/ml) in MQ water, per plant (56). Once infected, plants were incubated under standard conditions and wilting signs were recorded daily on a disease index scale from 0 (no wilt) to 4 (death). At 4, 8, and 12 dpi, bacterial multiplication in tomato plants was measured by collecting 3 cm of the stem, 0.5 cm above the petiole of the first true leaf, into tubes containing 300 μl of MQ water. After 30 min of incubation at room temperature, serial dilutions were plated in corresponding rich B medium to measure viable bacteria.

For petiole inoculation assays, 4-week-old plants were directly infected by inoculation with a 100-μl drop (approximately 10^3 CFU) of a sample obtained from a culture at OD_{600} of 0.1 (~10^8 CFU/ml) diluted in MQ water. Bacterial injections into the stem, above the petiole of the first true leaf, were performed using a sterile needle, and plants were incubated under standard conditions (56). Disease index scale values were assigned daily during 9 days, and at 3, 6, and 9 dpi, bacterial multiplication in tomato plants was measured as previously described by collection of 3 cm of the stem, 0.5 cm above the inoculation point, and plating in corresponding rich B plates.

Luminescence assays. Overnight cultures of all strains carrying *PhrpB*::*lux* were inoculated in MM supplemented with glutamate and gentamicin at absorbance of 0.3 (OD_{600}), and culture aliquots were collected at 0, 4, 6, and 8 h postinoculation to measure luminescence and absorbance (57). Expression of the *hrpB* promoter was represented as relative light units (RLU) per second. A FB-12 luminometer (Berthold Detection Systems, Pforzheim, Germany) and a V-1200 spectrophotometer (VWR, Radnor, PA) were used for each measurement, respectively.

To evaluate bacterial distribution through plant stem of reporter strains, 4-week-old plants were infected as previously described in a petiole inoculation assay but with slight modifications. Drops (10 μl) of cultures of each luminescent strain (approximately 10^3 CFU) were injected into the plant stem, above the petiole of the second true leaf. Once infected, plants were incubated under standard conditions and at 3 and 6 dpi, luminescence in tomato plants stem was measured by collecting 0.5 cm of internodes 2 and 3 into tubes containing 200 μl of MQ water. After 30 min of incubation, luminescence was measured from different stem cuts.

Statistics. All data were analyzed in a two-tailed, one-way analysis of variance (ANOVA) followed by the Tukey test for *post hoc* multiple-group comparisons. Results were considered statistically significant when a P value of <0.05 was obtained.

Data accessibility. The data that support the findings of this study are available from the corresponding author upon request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 1.3 MB.

FIG S2, TIF file, 2.5 MB.

FIG S3, TIF file, 0.5 MB.

FIG S4, TIF file, 1.5 MB.

FIG S5, TIF file, 0.5 MB.

FIG S6, TIF file, 1.1 MB.

FIG S7, TIF file, 0.5 MB.

TABLE S1, DOCX file, 0.01 MB.

ACKNOWLEDGMENTS

This study was funded by grants BIO2016-77011-R and AGL2016-78002-R (Spanish Ministry of Economy and Competitiveness). We acknowledge financial support from the Severo Ochoa Program for Centers of Excellence in R&D (SEV-2015-0533) and the CERCA Program from the Catalan Government (Generalitat de Catalunya). J.A. is a Serra Húnter Fellow (Generalitat de Catalunya). The project that gave rise to these results received the support of a fellowship (code is LCF/BQ/IN17/11620004) from la Caixa Foundation (identifier [ID] 100010434). This project has received funding from the European Union's Horizon 2020 research and innovation program under Marie Skłodowska-Curie grant agreement no. 713673.

We thank Joan Ruiz (Universitat Autònoma de Barcelona [UAB]) and Susana Escribano (UAB) for their excellent technical assistance, as well as UAB student Paula García for her helpful support.

M.V., J.A., N.S.C., and J.B. conceived the study. J.A. and J.C. performed the *in vitro* bacterial experiments. J.C. and P.S. performed all the bacterial infections *in planta*. M.V., J.A., N.S.C., and J.C. supervised the study. M.V., J.A., J.C., and P.S. analyzed the results. M.V., J.A., N.S.C., J.B., J.C., and P.S. wrote the article with contributions from all of us.

We declare that we have no conflict of interests.

REFERENCES

1. Peeters N, Guidot A, Vailleau F, Valls M. 2013. *Ralstonia solanacearum*, a widespread bacterial plant pathogen in the post-genomic era. Mol Plant Pathol 14:651–662. https://doi.org/10.1111/mpp.12038.
2. Allen C, Prior P, Hayward AC. 2005. Bacterial wilt disease and the Ralstonia solanacearum species complex. American Phytopathological Society Press, St. Paul, MN.
3. Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, Dow M, Verdier V, Beer SV, Machado MA, Toth I, Salmond G, Foster GD. 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. Mol Plant Pathol 13:614–629. https://doi.org/10.1111/j.1364-3703.2012.00804.x.
4. Caldwell D, Kim B-S, Iyer-Pascuzzi AS. 2017. *Ralstonia solanacearum* differentially colonizes roots of resistant and susceptible tomato plants. Phytopathology 107:528–536. https://doi.org/10.1094/PHYTO-09-16-0353-R.
5. Tans-Kersten J, Huang H, Allen C. 2001. *Ralstonia solanacearum* needs motility for invasive virulence on tomato. J Bacteriol 183:3597–3605. https://doi.org/10.1128/JB.183.12.3597-3605.2001.
6. Yao J, Allen C. 2006. Chemotaxis is required for virulence and competitive fitness of the bacterial wilt pathogen *Ralstonia solanacearum*. J Bacteriol 188:3697–3708. https://doi.org/10.1128/JB.188.10.3697-3708.2006.
7. Mattick JS. 2002. Type IV pili and twitching motility. Annu Rev Microbiol 56:289–314. https://doi.org/10.1146/annurev.micro.56.012302.160938.
8. Kang Y, Liu H, Genin S, Schell MA, Denny TP. 2002. *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. Mol Microbiol 46:427–437. https://doi.org/10.1046/j.1365-2958.2002.03187.x.
9. Liu H, Kang Y, Genin S, Schell MA, Denny TP, Genin S, Denny TP. 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. Microbiology 147:3215–3229. https://doi.org/10.1099/00221287-147-12-3215.
10. Bertrand JJ, West JT, Engel JN. 2010. Genetic analysis of the regulation of type IV pilus function by the Chp chemosensory system of *Pseudomonas aeruginosa*. J Bacteriol 192:994–1010. https://doi.org/10.1128/JB.01390-09.
11. Sampedro I, Parales RE, Krell T, Hill JE. 2015. *Pseudomonas* chemotaxis. FEMS Microbiol Rev 39:17–46. https://doi.org/10.1111/1574-6976.12081.
12. Bahar O, Levi N, Burdman S, Burdman S. 2011. The cucurbit pathogenic bacterium *Acidovorax citrulli* requires a polar flagellum for full virulence before and after host-tissue penetration. Mol Plant Microbe Interact 24:1040–1050. https://doi.org/10.1094/MPMI-02-11-0041.
13. Bahar O, Goffer T, Burdman S. 2009. Type IV pili are required for virulence, twitching motility, and biofilm formation of *Acidovorax avenae* subsp. *citrulli*. Mol Plant Microbe Interact 22:909–920. https://doi.org/10.1094/MPMI-22-8-0909.
14. Taguchi F, Ichinose Y. 2011. Role of type IV pili in virulence of *Pseudomonas syringae* pv. *tabaci* 6605: correlation of motility, multidrug resistance, and HR-inducing activity on a nonhost plant. Mol Plant Microbe Interact 24:1001–1011. https://doi.org/10.1094/MPMI-02-11-0026.
15. Monteiro F, Solé M, van Dijk I, Valls M. 2012. A chromosomal insertion toolbox for promoter probing, mutant complementation, and pathogenicity studies in *Ralstonia solanacearum*. Mol Plant Microbe Interact 25:557–568. https://doi.org/10.1094/MPMI-07-11-0201.
16. Wheatley RM, Poole PS. 2018. Mechanisms of bacterial attachment to roots. FEMS Microbiol Rev 42:448–461. https://doi.org/10.1093/femsre/fuy014.
17. Whitchurch CB, Leech AJ, Young MD, Kennedy D, Sargent JL, Bertrand JJ, Semmler ABT, Mellick AS, Martin PR, Alm RA, Hobbs M, Beatson SA, Huang B, Nguyen L, Commolli JC, Engel JN, Darzins A, Mattick JS. 2004. Characterization of a complex chemosensory signal transduction system which controls twitching motility in *Pseudomonas aeruginosa*. Mol Microbiol 52:873–893. https://doi.org/10.1111/j.1365-2958.2004.04026.x.
18. Zhou X, Qian G, Chen Y, Du L, Liu F, Yuen GY. 2015. PilG is involved in the regulation of twitching motility and antifungal antibiotic biosynthesis in the biological control agent *Lysobacter enzymogenes*. Phytopathology 105:1318–1324. https://doi.org/10.1094/PHYTO-12-14-0361-R.
19. Darzins A. 1994. Characterization of a *Pseudomonas aeruginosa* gene cluster involved in pilus biosynthesis and twitching motility: sequence similarity to the chemotaxis proteins of enterics and the gliding bacterium *Myxococcus xanthus*. Mol Microbiol 11:137–153. https://doi.org/10.1111/j.1365-2958.1994.tb00296.x.
20. Cursino L, Galvani CD, Athinuwat D, Zaini PA, Li Y, De la Fuente L, Hoch HC, Burr TJ, Mowery P. 2011. Identification of an operon, Pil-Chp, that controls twitching motility and virulence in *Xylella fastidiosa*. Mol Plant Microbe Interact 24:1198–1206. https://doi.org/10.1094/MPMI-10-10-0252.
21. Leech AJ, Mattick JS. 2006. Effect of site-specific mutations in different phosphotransfer domains of the chemosensory protein ChpA on *Pseudomonas aeruginosa* motility. J Bacteriol 188:8479–8486. https://doi.org/10.1128/JB.00157-06.
22. Fulcher NB, Holliday PM, Klem E, Cann MJ, Wolfgang MC. 2010. The *Pseudomonas aeruginosa* Chp chemosensory system regulates intracellular cAMP levels by modulating adenylate cyclase activity. Mol Microbiol 76:889–904. https://doi.org/10.1111/j.1365-2958.2010.07135.x.
23. Zhou M, Shen D, Xu G, Liu F, Qian G. 2017. ChpA controls twitching motility and broadly affects gene expression in the biological control agent *Lysobacter enzymogenes*. Curr Microbiol 74:566–574. https://doi.org/10.1007/s00284-017-1202-5.
24. Rosenberg T, Salam BB, Burdman S. 2018. Association between loss of type IV pilus synthesis ability and phenotypic variation in the cucurbit pathogenic bacterium *Acidovorax citrulli*. Mol Plant Microbe Interact 31:548–559. https://doi.org/10.1094/MPMI-12-17-0324-R.
25. Kilmury SLN, Burrows LL. 2018. The *Pseudomonas aeruginosa* PilSR two-component system regulates both twitching and swimming motilities. mBio 9:e01310-18. https://doi.org/10.1128/mBio.01310-18.
26. Brumbley SM, Denny TP. 1990. Cloning of wild-type *Pseudomonas solanacearum phcA*, a gene that when mutated alters expression of multiple traits that contribute to virulence. J Bacteriol 172:5677–5685. https://doi.org/10.1128/jb.172.10.5677-5685.1990.
27. Meng F, Yao J, Allen C. 2011. A MotN mutant of *Ralstonia solanacearum* is hypermotile and has reduced virulence. J Bacteriol 193:2477–2486. https://doi.org/10.1128/JB.01360-10.
28. Das A, Rangaraj N, Sonti RV. 2009. Multiple adhesin-like functions of *Xanthomonas oryzae* pv. *oryzae* are involved in promoting leaf attachment, entry, and virulence on rice. Mol Plant Microbe Interact 22:73–85. https://doi.org/10.1094/MPMI-22-1-0073.
29. Ryan RP, Fouhy Y, Lucey JF, Jiang B-L, He Y-Q, Feng J-X, Tang J-L, Dow JM. 2007. Cyclic di-GMP signalling in the virulence and environmental adaptation of *Xanthomonas campestris*. Mol Microbiol 63:429–442. https://doi.org/10.1111/j.1365-2958.2006.05531.x.
30. Narulita E, Addy HS, Kawasaki T, Fujie M, Yamada T. 2016. The involvement of the PilQ secretin of type IV pili in phage infection in *Ralstonia solanacearum*. Biochem Biophys Res Commun 469:868–872. https://doi.org/10.1016/j.bbrc.2015.12.071.
31. Siri MI, Sanabria A, Boucher C, Pianzzola MJ. 2014. New type IV pili–related genes involved in early stages of *Ralstonia solanacearum* potato infection. Mol Plant Microbe Interact 27:712–724. https://doi.org/10.1094/MPMI-07-13-0210-R.
32. Addy HS, Askora A, Kawasaki T, Fujie M, Yamada T. 2012. Through infection by φRSM filamentous phages. Phytopathology 102:469–477. https://doi.org/10.1094/PHYTO-11-11-0319-R.
33. Ray SK, Kumar R, Peeters N, Boucher C, Genin S. 24 March 2015, posting date. *rpoN1*, but not *rpoN2*, is required for twitching motility, natural competence, growth on nitrate, and virulence of *Ralstonia solanacearum*. Front Microbiol https://doi.org/10.3389/fmicb.2015.00229.
34. McCarthy Y, Ryan RP, O'Donovan K, He Y-Q, Jiang B-L, Feng J-X, Tang J-L, Dow JM. 2008. The role of PilZ domain proteins in the virulence of

Xanthomonas campestris pv. *campestris*. Mol Plant Pathol 9:819–824. https://doi.org/10.1111/j.1364-3703.2008.00495.x.
35. Shi X, Lin H. 8 April 2016, posting date. Visualization of twitching motility and characterization of the role of the PilG in *Xylella fastidiosa*. J Vis Exp https://doi.org/10.3791/53816.
36. Haiko J, Westerlund-Wikström B. 2013. The role of the bacterial flagellum in adhesion and virulence. Biology (Basel) 2:1242–1267. https://doi.org/10.3390/biology2041242.
37. Rossi FA, Medeot DB, Liaudat JP, Pistorio M, Jofré E. 2016. In *Azospirillum brasilense*, mutations in *flmA* or *flmB* genes affect polar flagellum assembly, surface polysaccharides, and attachment to maize roots. Microbiol Res 190:55–62. https://doi.org/10.1016/j.micres.2016.05.006.
38. Zheng H, Mao Y, Teng J, Zhu Q, Ling J, Zhong Z. 2015. Flagellar-dependent motility in *Mesorhizobium tianshanense* is involved in the early stage of plant host interaction: study of an *flgE* mutant. Curr Microbiol 70:219–227. https://doi.org/10.1007/s00284-014-0701-x.
39. Mhedbi-Hajri N, Jacques M-A, Koebnik R. 2011. Adhesion mechanisms of plant-pathogenic *Xanthomonadaceae*. Adv Exp Med Biol 715:71–89. https://doi.org/10.1007/978-94-007-0940-9_5.
40. Croes CL, Moens S, van Bastelaere E, Vanderleyden J, Michiels KW. 1993. The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots. J Gen Microbiol 139:2261–2269. https://doi.org/10.1099/00221287-139-9-2261.
41. Belas R. 2014. Biofilms, flagella, and mechanosensing of surfaces by bacteria. Trends Microbiol 22:517–527. https://doi.org/10.1016/j.tim.2014.05.002.
42. O'Toole GA, Kolter R. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 30:295–304. https://doi.org/10.1046/j.1365-2958.1998.01062.x.
43. Yao J, Allen C. 2007. The plant pathogen *Ralstonia solanacearum* needs aerotaxis for normal biofilm formation and interactions with its tomato host. J Bacteriol 189:6415–6424. https://doi.org/10.1128/JB.00398-07.
44. Miller JH. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
45. Boucher CA, Barberis PA, Demery DA. 1985. Transposon mutagenesis of *Pseudomonas solanacearum*: isolation of Tn5-induced avirulent mutants. Microbiology 131:2449–2457. https://doi.org/10.1099/00221287-131-9-2449.
46. Hendrick CA, Sequeira L. 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. Appl Environ Microbiol 48:94–101. https://doi.org/10.1128/AEM.48.1.94-101.1984.
47. Powell S, Forslund K, Szklarczyk D, Trachana K, Roth A, Huerta-Cepas J, Gabaldón T, Rattei T, Creevey C, Kuhn M, Jensen LJ, von Mering C, Bork P. 2014. eggNOG v4.0: nested orthology inference across 3686 organisms. Nucleic Acids Res 42:D231–D239. https://doi.org/10.1093/nar/gkt1253.
48. Yu J-H, Hamari Z, Han K-H, Seo J-A, Reyes-Domínguez Y, Scazzocchio C. 2004. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet Biol 41:973–981. https://doi.org/10.1016/j.fgb.2004.08.001.
49. Monteiro F, Genin S, van Dijk I, Valls M. 2012. A luminescent reporter evidences active expression of *Ralstonia solanacearum* type III secretion system genes throughout plant infection. Microbiology 158:2107–2116. https://doi.org/10.1099/mic.0.058610-0.
50. Raza W, Ling N, Yang L, Huang Q, Shen Q. 2016. Response of tomato wilt pathogen *Ralstonia solanacearum* to the volatile organic compounds produced by a biocontrol strain *Bacillus amyloliquefaciens* SQR-9. Sci Rep 6:24856. https://doi.org/10.1038/srep24856.
51. Adler J. 1973. A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. J Gen Microbiol 74:77–91. https://doi.org/10.1099/00221287-74-1-77.
52. Bertolla F, Van Gijsegem F, Nesme X, Simonet P. 1997. Conditions for natural transformation of *Ralstonia solanacearum*. Appl Environ Microbiol 63:4965–4968. https://doi.org/10.1128/AEM.63.12.4965-4968.1997.
53. Paola A, Cruz Z, Ferreira V, Pianzzola MJ, Siri MI, Coll NS, Valls M. 2014. A novel, sensitive method to evaluate potato germplasm for bacterial wilt resistance using a luminescent *Ralstonia solanacearum* reporter strain. Mol Plant Microbe Interact 27:277–285. https://doi.org/10.1094/MPMI-10-13-0303-FI.
54. O'Toole GA, Kolter R. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 28:449–461. https://doi.org/10.1046/j.1365-2958.1998.00797.x.
55. Yang W-C, Lin Y-M, Cheng Y-S, Cheng C-P. 2013. *Ralstonia solanacearum RSc0411* (*lptC*) is a determinant for full virulence and has a strain-specific novel function in the T3SS activity. Microbiology 159:1136–1148. https://doi.org/10.1099/mic.0.064915-0.
56. Morel A, Peeters N, Vailleau F, Barberis P, Jiang G, Berthomé R, Guidot A. 2018. Plant pathogenicity phenotyping of *Ralstonia solanacearum* strains. Methods Mol Biol 1734:223–239. https://doi.org/10.1007/978-1-4939-7604-1_18.
57. Puigvert M, Solé M, López-Garcia B, Coll NS, Beattie KD, Davis RA, Elofsson M, Valls M. 2019. Type III secretion inhibitors for the management of bacterial plant diseases. Mol Plant Pathol 20:20–32. https://doi.org/10.1111/mpp.12736.

Metadata of the chapter that will be visualized online

Chapter Title	Identification of Type III Secretion Inhibitors for Plant Disease Management	
Copyright Year	2021	
Copyright Holder	Springer Science+Business Media, LLC, part of Springer Nature	
Author	Family Name	**Pedro Jové**
	Particle	**de**
	Given Name	**Roger**
	Suffix	
	Division	Department of Genetics
	Organization	University of Barcelona
	Address	Barcelona, Catalonia, Spain
	Organization	Centre for Research in Agricultural Genomics (CSIC-IRTA-UAB-UB)
	Address	Bellaterra, Catalonia, Spain
Author	Family Name	**Sebastià**
	Particle	
	Given Name	**Pau**
	Suffix	
	Organization	Centre for Research in Agricultural Genomics (CSIC-IRTA-UAB-UB)
	Address	Bellaterra, Catalonia, Spain
Corresponding Author	Family Name	**Valls**
	Particle	
	Given Name	**Marc**
	Suffix	
	Division	Department of Genetics
	Organization	University of Barcelona
	Address	Barcelona, Catalonia, Spain
	Organization	Centre for Research in Agricultural Genomics (CSIC-IRTA-UAB-UB)
	Address	Bellaterra, Catalonia, Spain
	Email	marcvalls@ub.edu

Abstract

Bacterial plant pathogens are among the most devastating threats to agriculture. To date, there are no effective means to control bacterial plant diseases due to the restrictions in the use of antibiotics in agriculture. A novel strategy under study is the use of chemical compounds that inhibit the expression of key bacterial virulence determinants. The type 3 secretion system is essential for virulence of many Gram-negative bacteria because it injects into the plant host cells bacterial proteins that interfere with their immune system. Here, we describe the methodology to identify bacterial

type 3 secretion inhibitors, including a series of protocols that combine *in planta* and in vitro experiments. We use *Ralstonia solanacearum* as a model because of the number of genetic tools available in this organism and because it causes bacterial wilt, one of the most threatening plant diseases worldwide. The procedures presented can be used to evaluate the effect of different chemical compounds on bacterial growth and virulence.

Keywords (separated by '-') Bacterial plant pathogens - Type 3 secretion system - *Ralstonia solanacearum* - Chemical inhibitors - Plants - Protocols - Immunodetection - In vitro inhibitory test

Chapter 4

Identification of Type III Secretion Inhibitors for Plant Disease Management

Roger de Pedro Jové, Pau Sebastià, and Marc Valls

Abstract

Bacterial plant pathogens are among the most devastating threats to agriculture. To date, there are no effective means to control bacterial plant diseases due to the restrictions in the use of antibiotics in agriculture. A novel strategy under study is the use of chemical compounds that inhibit the expression of key bacterial virulence determinants. The type 3 secretion system is essential for virulence of many Gram-negative bacteria because it injects into the plant host cells bacterial proteins that interfere with their immune system. Here, we describe the methodology to identify bacterial type 3 secretion inhibitors, including a series of protocols that combine *in planta* and in vitro experiments. We use *Ralstonia solanacearum* as a model because of the number of genetic tools available in this organism and because it causes bacterial wilt, one of the most threatening plant diseases worldwide. The procedures presented can be used to evaluate the effect of different chemical compounds on bacterial growth and virulence.

Key words Bacterial plant pathogens, Type 3 secretion system, *Ralstonia solanacearum*, Chemical inhibitors, Plants, Protocols, Immunodetection, In vitro inhibitory test

1 Introduction

Bacteria can cause a range of diseases in economically important crops, leading to important losses. *Ralstonia solanacearum*, the causal agent of bacterial wilt, is one of the most devastating plant pathogens worldwide. The lack of effective means to control bacterial diseases and block the spread of these pathogens urge for new control strategies. The use of antibiotics and copper-based compounds is nowadays banned or tightly regulated in many countries [1, 2]. Using compounds that inhibit specific bacterial virulence factors is a promising and sustainable strategy.

The type 3 secretion system (T3SS) is one of the most distinctive hallmarks of Gram-negative bacterial pathogens. These pathogens use the T3SS to inject small molecules called effectors inside

Roger de Pedro Jové and Pau Sebastià contributed equally to this work.

Glenn R. Hicks and Chunhua Zhang (eds.), *Plant Chemical Genomics: Methods and Protocols*, Methods in Molecular Biology, vol. 2213, https://doi.org/10.1007/978-1-0716-0954-5_4, © Springer Science+Business Media, LLC, part of Springer Nature 2021

the plant cell. Bacterial effectors hijack plant defense mechanisms and manipulate different metabolic pathways to successfully colonize the host [3]. Mutant bacteria devoid of the T3SS are totally nonpathogenic so that a possible strategy to inhibit bacterial virulence is to use chemical compounds that block the expression of this secretion system and impede bacterial colonization throughout the plant [4–6].

In this protocol, we present a stepwise guide to assess the ability of different chemical compounds to transcriptionally downregulate the expression of key T3SS genes and to test if they could be used as a means to decrease the virulence of the tested pathogens *in planta*.

2 Materials

2.1 Plant Growth

1. *Nicotiana benthamiana*; *Nicotiana tabacum*; and *Solanum lycopersicum* cv. Marmande.
2. Soil mix: Peat soil substrate n°2 + vermiculite + perlite (*see* **Note 1**).
3. Plant growth chambers with temperature, humidity, and photoperiod control.

2.2 Bacterial Strains and Growth

1. *Ralstonia solanacearum* GMI1000 reporter strains for transcription of *hrpB* (*PhrpB*::*luxCDABE*), *psbA* (*PpsbA*::*luxCDABE*), and *hrpY* (*PhrpY*::*luxCDABE*). *R. solanacearum* GMI1000 *PpsbA*::*avrA*-HA.
2. B medium: 10 g/L bacteriological peptone, 1 g/L yeast extract, and 1 g/L casamino acids. Add 1.5% agar for solid media before autoclaving. Before plating, add 0.5% glucose and 0.005% triphenyltetrazolium chloride (TTC). Supplement with the appropriate antibiotics (*see* **Notes 2** and **3**).
3. Boucher's minimal medium [7]: To prepare 1 L of 2× Boucher's medium, mix 100 mL of 5× M63 medium (10 g/L $(NH_4)_2SO_4$, 68 g/L KH_2PO_4, and 2.5 mg/L $FeSO_4{\cdot}7H_2O$, pH 7 with KOH) with 405 µL of 1 M $MgSO_4{\cdot}7H_2O$ and adjust to 1 L with sterile distilled water. Before use, dilute to 1× with sterile distilled water (or 2× agar on water for plates). Supplement with 20 mM glutamate and appropriate antibiotics.

2.3 T3SS Inhibition Test In Vitro

1. Potential type 3 secretion inhibitory compound to test.
2. DMSO.
3. Incubator at 28 °C with rotor.
4. Luminometer.
5. Spectrophotometer.

2.4 Effect of the Tested Compound on Bacterial T3E Secretion

1. Sucrose.
2. Congo red.
3. 0.22-μM filter.
4. 10-mL syringe.
5. 25% trichloroacetic acid.
6. 90% acetone.
7. Phosphate-buffered saline (PBS) 1×: 8 g/L NaCl, 0.201 g/L KCl, 1.42 g/L Na_2HPO_4, 0.272 g/L KH_2PO_4.
8. 4× Laemmli buffer.
9. Digital sonifier.
10. Primary anti-HA rat monoclonal antibody conjugated to horseradish peroxidase (HRP) in Tris-buffered saline (TBS) with 0.1% Tween-20 and 1% skimmed milk (*see* **Note 4**).
11. Coomassie blue.
12. LAS-4000 mini system.

2.5 In Planta Experiments

1. Blunt-end syringe.
2. 100% ethanol.
3. Leaf disk puncher.
4. Potter S homogenizer.

3 Methods

3.1 Plant and Bacterial Growth

3.1.1 N. benthamiana/N. tabacum

1. Sow *N. benthamiana* or *N. tabacum* seeds in a pot at 26 °C and 14 h light/10 h darkness.
2. After 10 days, transfer each seedling to individual pots.
3. After 10 days, transfer each individual plant to single big pots. These plants will be ready for assays after 3 weeks (*see* **Notes 5** and **6**).

3.1.2 Solanum lycopersicum *cv. Marmande*

1. Sterilize Marmande tomato seeds with a sterile solution containing 1:3.33 of commercial bleach (4.7% concentrated) and 0.05% triton. Keep the seeds in the solution for 10 min. Wash with sterile distilled water at least five times.
2. Sow the sterilized seeds and cover with plastic film.
3. Keep the plants in the growth chamber at 22 °C, 16 h light and 8 h darkness for 1 week, until tomato seedlings emerge and touch the plastic film on top.
4. Transfer each tomato seedling to individual soil pots with the soil mix and let them grow for 3 weeks in a chamber at 22 °C and 16 h light and 8 h darkness (*see* **Note 5**).

3.1.3 Ralstonia Solanacearum

1. Streak the bacterial strain from a glycerol stock at −80 °C on B medium supplemented with antibiotics for 2 days at 28 °C.
2. Pick a single colony and incubate in liquid B or minimal media.

3.2 In Vitro T3SS Inhibitor Screening in Ralstonia Solanacearum

1. Grow an overnight pre-culture in liquid B media supplemented with antibiotics (*see* **Notes 7** and **8**).
2. Centrifuge the overnight pre-culture in 2-mL Eppendorf tubes at RT for 1 min at maximum speed, discard the supernatant, and resuspend the bacterial pellet in 1 mL of sterile distilled H_2O.
3. Measure the OD_{600} with a spectrophotometer (*see* **Note 9**).
4. Adjust to a final OD_{600} of 0.3 adding the right pre-culture volume to a culture tube containing 1.5 mL of fresh Boucher's minimal medium supplemented with 20 mM glutamate, antibiotic, and 100 mM inhibitory test compound/DMSO (*see* **Note 10**).
5. Mix by vortexing for a few seconds and incubate in a shaker.
6. Measure luminescence at times 0, 4, 6, 8 and 24 h transferring 200 µL from each tube into a 1.5-mL Eppendorf tube and quantifying light emission from the reporter in the luminometer. For each time point, measure as well OD_{600} in a spectrophotometer by transferring the 200 µL into a cuvette containing 800 µL of distilled water (*see* **Notes 11–13**).

3.3 Effect of the Tested Compound on Bacterial T3E Secretion

1. From an overnight culture of liquid B medium supplemented with antibiotics, adjust to a final OD of 0.2 (2×10^8 CFUs/mL) in a final volume of 10 mL of minimal medium supplemented with antibiotics, 10 mM glutamate, 10 mM sucrose, 100 µg/mL congo red (*see* **Note 14**), and 100 µg/mL of the test inhibitor compound (or 10 µL of DMSO as a control).
2. Incubate at room temperature for 12–14 h (or until OD_{600} reaches 1).
3. Transfer the culture to a 50-mL falcon tube and centrifuge at 4000 × *g* for 10 min.
4. Filter the supernatant through a 0.22-µM filter with a syringe in order to remove any bacteria. The bacterial pellet is also kept at −20 °C for further analysis.
5. Add 10 mL of cold 25% TCA to the filtered supernatant and let it precipitate all night long at 4 °C.
6. Centrifuge at 6000 × *g* for 30 min at 4 °C and discard the supernatant.
7. Wash the protein pellet (it will contain all secreted proteins in the medium) twice with cold 90% acetone and let it dry at RT.

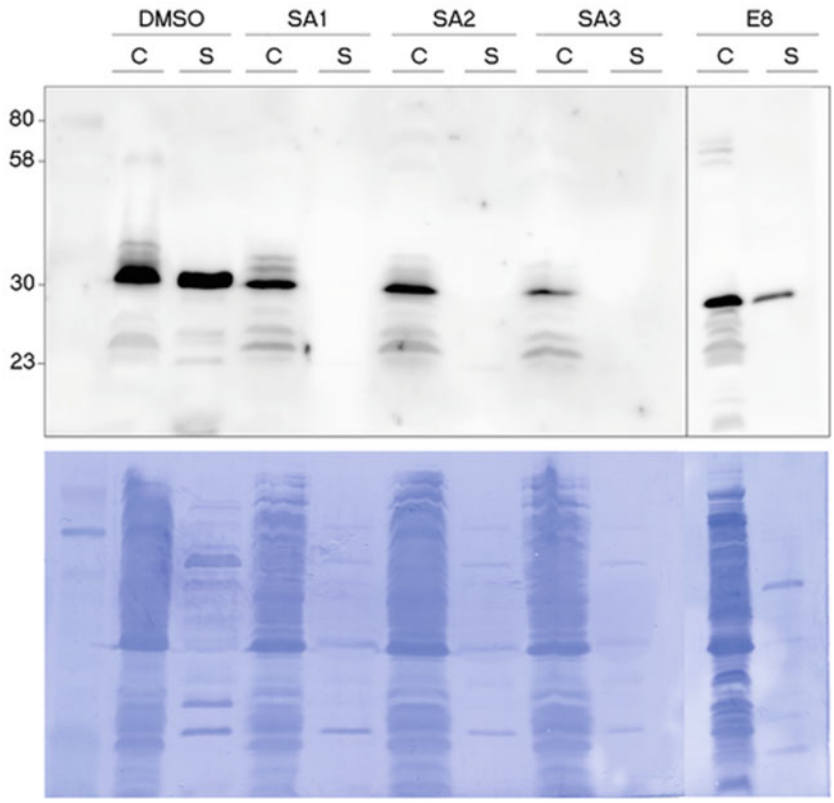


Fig. 1 Immunoblot of the secreted T3 effector (in this case, AvrA-HA) after treatment with four different inhibitory compounds (SA1-3 and E8) or the control (DMSO). The cytosolic (C) and secreted (S) fractions were separated by centrifugation. The protein of interest was detected with anti-HA antibody. Coomassie blue-stained membranes (below) used in the western blotting are also shown. (Reproduced from [9] with permission of John Wiley and Sons)

8. Resuspend the protein pellet in 100 µL of PBS 1×. Mix 15 µL of this solution with 15 µL of Laemmli buffer.
9. Recover the frozen bacterial pellet, freeze–thaw 3–4 times (−80 °C—RT cycles), resuspend in 1 mL of 1× PBS, and sonicate the cells using a sonifier (*see* **Note 15**). Mix 15 µL of the mixture with 15 µL of Laemmli buffer.
10. Boil the samples for 5 min and load it onSDS-PAGE (it will be a 100× concentration from initial culture).
11. The presence of particular proteins in the extracts can be analyzed by immunoblot using an antibody against the protein of interest. Coomassie-stained sodium dedecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) membranes can be visualized using a LAS-4000 mini system (*see* Fig. 1).

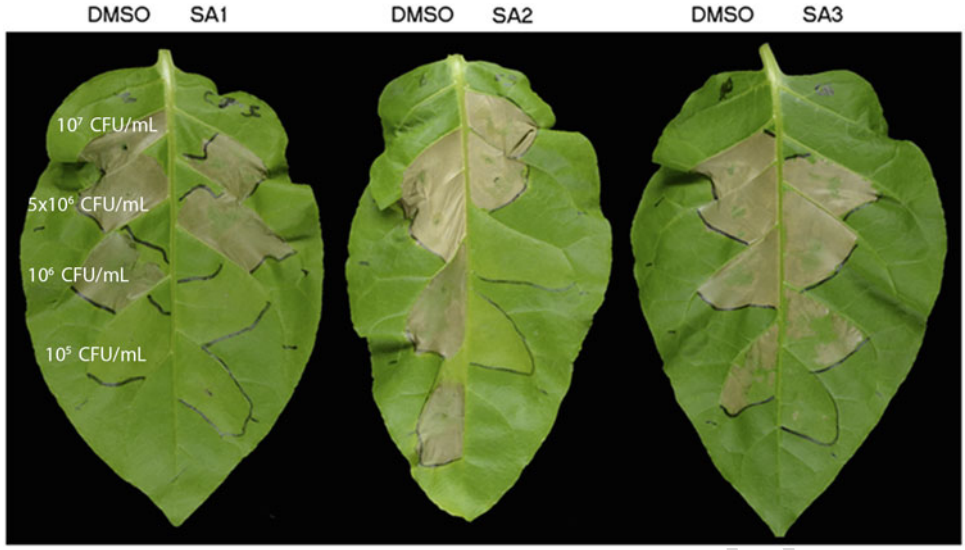


Fig. 2 *N. benthamiana* leaves infiltrated with serial dilutions of *R. solanacearum* preincubated with inhibitory compounds (in this case, SA1, SA2, SA3) or with a control solution (DMSO). Leaves were photographed 2 days post-infiltration. (Reproduced from [9] with permission of John Wiley and Sons)

3.4 In Vivo T3E Translocation Test Using Hypersensitive Response Assays

1. To the overnight culture of the desired bacterial strain (e.g., *R. solanacearum* GMI1000) in Boucher's minimal medium supplemented with 20 mM glutamate and antibiotic, add the tested inhibitory compound at 100 μM (or with DMSO for the nontreated condition) and incubate for 8 h.
2. Centrifuge at maximum speed for 8 min and discard the supernatant.
3. Resuspend bacterial pellet with sterile distilled water and measure the OD. Make serial dilutions ranging from 10^7 to 10^5 CFUs/mL (*see* **Note 16**).
4. Leaf-infiltrate *N. benthamiana* and *N. tabacum* plants with a blunt-end syringe following a predesigned scheme (*see* **Note 17** and Fig. 2).
5. The first signs of hypersensitive responses are visible 12 h post-infiltration, but they can be better appreciated when the dead tissue is totally dry, so the pictures are taken 2 days post-infiltration in *N. tabacum*, and 5 days post-infiltration in *N. benthamiana* (*see* **Note 18**).

3.5 Compound Effect on Bacterial Fitness In Planta

1. Grow an overnight pre-culture in liquid B medium supplemented with antibiotics.
2. Measure the OD_{600} of the pre-culture and adjust a bacterial suspension to 10^5 CFU/mL (OD_{600} = 0.0001) with autoclaved tap water supplemented with each test compound at 100 μM (or DMSO alone for control condition).

3. Hand-infiltrate 4 tomato leaves per tested compound with a blunt-end syringe (*see* **Note 19**).
4. Place the infiltrated plants in the growth chamber for 1 h at 27 °C and 60% relative humidity.
5. At time 0 (just after infiltration) and at 4 days post-infiltration (d.p.i.), collect 2 leaf discs (5 mm diameter) from the infiltrated area of six independent leaves. Combine in a 1.5-mL Eppendorf tube the disks from 2 leaves (4 disks total) to generate three biological replicates.
6. Homogenize the plant material with a Potter S homogenizer in 200 µL of sterile distilled water (*see* **Note 20**).
7. Add 800 µL of sterile distilled water to each Eppendorf tube.
8. Place the plants back in the growth chamber.
9. Prepare tenfold dilutions from the leaf homogenates (*see* **Note 21**).
10. Plate 10 µL drops of the 4 dilutions on plates of B medium (containing TTC and glucose) supplemented with antibiotics and incubate at 28 °C for 1–2 days to count colonies (*see* **Note 22**).

3.6 Effect of the T3 Secretion Inhibitor on Bacterial Virulence to Plants

1. Grow an overnight pre-culture in liquid B medium supplemented with antibiotics.
2. For each treatment, wound the roots of 12 plants grown in independent pots with a 1-mL pipette tip by making 4 holes in the soil around the stem. Water each plant with 40 mL of a bacterial suspension containing 10^8 CFUs/mL supplemented with 100 µM of the compound to test or DMSO (*see* **Note 23**).
3. Record wilting symptoms during 9 days after infection for each plant using a semiquantitative scale ranging from 0 (no wilting) to 4 (death) (*see* **Note 24**).

4 Notes

1. For 24 individual square pots mix: 7 L of peat soil, 0.2 L of vermiculite, and 0.2 L of perlite.
2. For gentamicin and tetracycline, use half of the recommended concentration in liquid media (e.g., 10 µg/mL gentamicin in solid medium and 5 µg/mL in liquid medium).
3. Keep the TTC solution and tetracycline away from direct light contact. Glucose strongly enhances exopolysaccharide production and TTC turns red through bacterial metabolism, so wild-type *R. solanacearum* colonies appear red with a thick mucus

halo in this medium. Spontaneous nonmucous mutants (usually rare) are nonpathogenic and can be discarded.

4. The anti-HA antibody (clone 3F10) from Roche, Switzerland, works well for us at 1:4000 dilution. Anti-HA antibodies from our resources might work as well, and we recommend testing for ideal dilutions before use.
5. To acclimate the plants, 2 days prior to bacterial inoculation, transfer them to the infection growth chamber (27 °C and 60% humidity).
6. For HR assays, plants should not be stressed. Clear signs of stress are chlorotic leaves and flowering. To avoid this, do not water in excess, and always use high-intensity light. Plants can be grown at 24–26 °C without any difference.
7. Minimal medium is appropriate when type 3 secretion gene expression has to be induced (e.g., *PhrpY::lux*). B medium is appropriate when high growth is desired, or expression of the type 3 secretion genes has to be repressed.
8. Normally, 10 mL of overnight culture should be enough to prepare 20 tubes for the inhibition test.
9. We recommend measuring OD_{600} from 1/10 dilutions of overnight cultures to avoid saturation, as spectrophotometers usually measure linearly between 0.01 and 2.
10. To ease the experiment, prepare these minimal media culture tubes the day before and store at 4 °C. Pre-warm the media before use.
11. Use a cuvette with the same growth medium as blank to calibrate the spectrophotometer.
12. This protocol can be scaled up to 96-well plates in case a larger set of inhibitors has to be tested. For growth measurements, a transparent bottom plate must be used. For luminescence measurements, use white opaque plates, which help reflecting luminescence and amplify the signal. The 96-well plates can be measured using a Spectramax M3 from Molecular Devices.
13. Luminescence measurements allow quantification of the transcriptional output at different time points, and OD_{600} measurements quantify bacterial growth to normalize luminescence per cell and rule out eventual inhibitory or bacteriostatic effects of the tested compounds.
14. Congo red enhances bacterial protein secretion through the type 3 secretion system [8].
15. We normally sonicate for 90s at 30% amplification and 10s ON/OFF intervals using a digital sonifier, Model 250/450 (BRANSON, USA). The required sonication time and intervals can vary for different sonifiers.

16. In *R. solanacearum*, an $OD_{600} = 1$ usually corresponds to 10^9 CFUs/mL.
17. It is recommended to randomize the infiltration of the bacterial dilutions in different leaves in order to get rid of eventual position effects. Infiltrate in the inter-vein areas to avoid a mixture of treatments.
18. For a better HR cell death visualization, the treated leaves can be bleached using 100% ethanol in a water bath at 60 °C for 20 min.
19. Tomato plants can be vacuum-infiltrated instead using Silwett as an adjuvant to facilitate infiltration (80 μL/L). Usually, 20–30 s of vacuum infiltration is enough per tomato plant, but timings might change in other plant species depending on the hardness of their leaves. A change in the leaf color to dark green indicates proper vacuum infiltration.
20. We use the mechanic drill with a plastic pestle, but a tissue lyser with beads or a classical mortar can also be used.
21. To ease manipulation, it is advisable to perform dilutions in 96-well plates using a multichannel pipette by transferring 10 μL into 90 μL of sterile distilled H_2O consecutively. Make sure to mix well each dilution.
22. For colony count, make sure that colonies are well separated. Bacterial growth is calculated as recovered CFU/cm^2 (area depends on the size of the leaf disk puncher).
23. In order to facilitate plant infection, it is better to stop watering them 2 days prior to inoculation.
24. Wilting symptoms are recorded based on a scale from 0 to 4: 0 = no wilting, 1 = 25% of the leaves wilted, 2 = 50% of the leaves wilted, 3 = 75% of the leaves wilted, and 4 = 100% of the leaves wilted. It is recommended that the same person carries out the whole symptom recording to avoid biases.

References

1. Duffy B, Schärer HJ, Bünter M et al (2005) Regulatory measures against Erwinia amylovora in Switzerland. EPPO Bull 35:239–244. https://doi.org/10.1111/j.1365-2338.2005.00820.x
2. MacKie KA, Müller T, Kandeler E (2012) Remediation of copper in vineyards—a mini review. Environ Pollut 167:16–26. https://doi.org/10.1016/j.envpol.2012.03.023
3. Büttner D (2016) Behind the lines-actions of bacterial type III effector proteins in plant cells. FEMS Microbiol Rev 40:894–937. https://doi.org/10.1093/femsre/fuw026
4. Hudson DL, Layton AN, Field TR et al (2007) Inhibition of type III secretion in *Salmonella enterica* serovar typhimurium by small-molecule inhibitors. Antimicrob Agents Chemother 51:2631–2635. https://doi.org/10.1128/AAC.01492-06
5. Kauppi AM, Nordfelth R, Uvell H et al (2003) Targeting bacterial virulence: inhibitors of type III secretion in Yersinia. Chem Biol 10:241–249. https://doi.org/10.1016/s1074-5521(03)00046-2
6. Muschiol S, Bailey L, Gylfe A et al (2006) A small-molecule inhibitor of type III secretion inhibits different stages of the infectious cycle

of chlamydia trachomatis. Proc Natl Acad Sci U S A 103:14566–14571. https://doi.org/10.1073/pnas.0606412103

7. Boucher CA, Barberis PA, Demery DA (1985) Transposon mutagenesis of pseudomonas solanacearum: isolation of Tn5-induced Avirulent mutants. Microbiology 131:2449–2457. https://doi.org/10.1099/00221287-131-9-2449

8. Bahrani FK, Sansonetti PJ, Parsot C (1997) Secretion of Ipa proteins by Shigella flexneri: inducer molecules and kinetics of activation. Infect Immun 65:4005–4010

9. Solé M, Puigvert M, Davis RA et al (2018) Type III secretion inhibitors for the management of bacterial plant diseases. Mol Plant Pathol 20:20–32. https://doi.org/10.1111/mpp.12736

Author Queries

Chapter No.: 4 467626_2_En

Query Refs.	Details Required	Author's response
AU1	Throughout the text, the term "Type 3" is used; however, in the title, the term "Type III" is used. Please check and ensure that this term is used in one way in this chapter.	
AU2	Please check whether the author names and affiliations are correct.	