UNIVERSIDAD
POLITECNICA
DE VALENCIA

BIOLOGY OF *Ralstonia solanacearum* PHYLOTYPE II IN HOST AND NON-HOST ENVIRONMENTS

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VALENCIA (SPAIN), 2009

This PhD Thesis was performed in the Instituto Valenciano de Investigaciones Agrarias (IVIA) in collaboration with the Universidad de Valencia, Estudio General.

The cover picture belongs to Chapter 2.1

UNIVERSIDAD POLITÉCNICA DE VALENCIA
Escuela Técnica Superior de Ingenieros Agrónomos
Dpto. de Biotecnología

INSTITUTO VALENCIANO DE INVESTIGACIONES AGRARIAS
Centro de Protección Vegetal y Biotecnología
Laboratorio de Bacteriología

En colaboración con la
UNIVERSIDAD DE VALENCIA, ESTUDIO GENERAL
Dpto. de Microbiología y Ecología

BIOLOGY OF *Ralstonia solanacearum* PHYLOTYPE II IN HOST AND NON-HOST ENVIRONMENTS

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Para optar al grado de:
DOCTOR CON MENCIÓN EUROPEA POR LA UNIVERSIDAD POLITÉCNICA DE VALENCIA
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Valencia, 2009

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CERTIFICAN:

Que Dª María Belén Álvarez Ortega ha realizado bajo su dirección, en el Laboratorio de Bacteriología del Centro de Protección Vegetal y Biotecnología del Instituto Valenciano de Investigaciones Agrarias, el trabajo que, con el título "Biology of *Ralstonia solanacearum* phylotype II in host and non-host environments", presenta para optar al grado de Doctor con Mención Europea por la Universidad Politécnica de Valencia.

Para que así conste a los efectos oportunos, firman el siguiente certificado en Valencia, a 21 de julio de 2009.

Dª María Milagros López González Dª Elena González Biosca

A mi abuela materna,
que siempre quiso ir a la Universidad……

Agradecimientos

Bueno, parece mentira que ya estemos aquí, en los agradecimientos.

En primer lugar, me gustaría dar las gracias al Instituto Valenciano de Investigaciones Agrarias y a su Director, Florentino Juste, por la beca de Doctorado que me fue concedida y con la que he podido realizar esta Tesis.

Claramente tengo todo que agradecer a mis Directoras, las Dras. María Milagros López (Chiqui) y Elena González Biosca. Además de que esta Tesis ha sido posible gracias a ellas, es que me han aguantado estoicamente durante ocho años… y, hasta el momento, todavía no he salido volando por la ventana del laboratorio hacia la pinada!!!! Esto sólo es mérito de ellas. A Chiqui le agradezco que me permitiera formar parte del equipo de investigación que dirige, y tomar un poco de contacto con el mundo de la Fitobacteriología que conoce tan bien. De ella he aprendido muchas cosas, y confieso que admiro su capacidad de trabajo, dedicación y responsabilidad, que son imposibles de igualar. A Elena le agradezco que haya sabido transmitirme pasión por la investigación y confianza en mi trabajo, en el que ella ha creído siempre más que yo misma, y le agradezco especialmente que siempre haya estado ahí, y que haya podido pedirle ayuda incluso los domingos a última hora (y lo he hecho, sí).

Quiero agradecer también a la que ha sido una familia para mí durante todo este período en Bacteriología.

A Clara y a Carmina, dos profesionales impresionantes y maravillosas personas, por ser tan buenas compañeras de trabajo y hacerme sentir como en casa desde el primer día de laboratorio. A Raquel, siempre llena de energía positiva y buenas vibraciones, por todas las conversaciones y las risas de 40 segundos entre pitido y pitido del timer. A Javi, impecable tanto en su manera de ser como de vestir, porque es una delicia su trato en cualquier situación y por ser tan comprensivo. A José Miguel, el xiquet, por haber hecho mucho más llevaderos los largos años de tesis en horarios imposibles que hemos compartido.

A Pablo, ese personaje de cómic (como le llamaba Raquel por sus gestos y miradas), por tantos años de amistad (también de discusiones) dentro y fuera del laboratorio. A Ramón, mi jefe *Agrobacterium*, que

siempre aparece cuando menos te lo esperas y desaparece cuando más lo esperas, y lo esperas, y lo esperas…., por sus conversaciones siempre animadas sobre la K84 y sus mutantes, y por haberse portado tan bien conmigo. A Ester, compañera *Ralstonia* de algún tiempo, por los buenos momentos pasados juntas y, la verdad, siento haberme dormido en todos aquellos viajes a Teruel….

A Morteza, esa cosita, por ser siempre tan cariñoso, tan buen compañero, y por estar siempre dispuesto a animarme y a hacerme bromas. A Silvia, otro encanto de persona, por ser tan atenta y por todas las veces que me ha ofrecido sinceramente su ayuda. A Ana, la simpatía personificada, no sé si le habrá sido efectivo el pimiento que le pasé… (a mí me lo pasó Carmina…). A Mónica, la "kuki", y a Jaime, el hombre silencioso, por su buen trato en el laboratorio. A Begoña, por su amabilidad y buen compañerismo. A Inma y a Jorge, por su simpatía.

A Silvia Martín, mi primera compañera en Bacteriología (entramos con una semana de diferencia), por todos los ratos de laboratorio y de sala de becarios de aquella época. También, por supuesto, a Paola y a Victoria, por todas las aventuras y los buenos momentos, que han sido muchos afortunadamente, que hemos pasado juntas durante todo este tiempo, tanto en el IVIA como fuera, aquí en Valencia y también en Sicilia.

A aquellas personas que han pasado por Bacteriología y que, además de ser buenos compañeros, me han dado muchos ánimos para acabar la tesis y han sido muy cariñosos conmigo, especialmente Margarita, Hélène, Leandro, Giuseppe, Ali Rouma y Elena Fhürer.

Cómo no, tengo mucho que agradecer a "los de enfrente" (Laboratorio de Virología-Inmunología), y no sólo por todas las puntas amarillas, tubos Eppendorf y matraces limpios que he tomado prestados… (y que he devuelto!!). Principalmente, a Mariano Cambra, por ser tan simpático y distendido, por sus bromas y su buen humor; a Maite y a Mari Carmen, por haber estado ahí, dispuestas a animarme y ayudarme en momentos de dificultad, lo que les agradezco especialmente a las dos; a Edson, por su amabilidad, buen compañerismo, su ayuda con los termocicladores…. y, por supuesto, sus caipirinhas!!; a Antonio, por su trato cariñoso, y especialmente a él y a su mujer por cuidarme en aquel

congreso del que no recuerdo nada…. siento haberos dado la noche!!; a Arantxa y a Nieves, por transmitir su alegría y el "buen rollo" que las caracteriza y que espero no pierdan nunca; a Eduardo, por todos sus ánimos constantes y por su sencillez de trato; a Anabel, por ser tan cercana y por su simpatía y, por supuesto, a Tere, porque siempre ha sido muy amable y accesible. También a Olga Esteban, que fue de las primeras personas en hacerme ver lo que podía ser una tesis, a Marta Patricia, por todas esas historias increíbles y divertidas que nos contaba, y por sus conocimientos de anatomía vegetal; y a Jaime (cariñosamente, "el de las moscas"), por sus bromas conmigo y su simpatía natural.

Especialmente a Consuelo y a Carmen, que me han acompañado durante toda mi etapa de cocina, y me han enseñado tantas cosas que no he podido incluir en esta Tesis, pero que están ahí.

Del Laboratorio de Virología, me gustaría agradecer en primer lugar a Pedro Moreno su simpatía y cordialidad. También a Susana (Susani), su amistad dentro y fuera del IVIA, las comidas y conversaciones de sobremesa en la sala de becarios, las salidas de fin de semana (junto con Pablo y Victoria)… tenías razón, la tesis se acaba, sí; a Patricia (Pata), sus risas, su sentido del humor, su buen compañerismo en el laboratorio y que sea un encanto de persona; a Manolo (Señor Manolo, como le llamaba una antigua jefa nuestra), sus conversaciones entrecortadas entre idas y venidas al laboratorio y sus bromas; a Silvia Ambrós, su ayuda cuando la he necesitado y sus consejos y ánimos; a Marta y a David, su simpatía y cordialidad. Por supuesto, también a Mari, su amabilidad y su buen compañerismo interlaboratorio, y a Reme, su simpatía y facilidad de trato.

Más allá de este pasillo del IVIA, hay otras personas a las que me gustaría mencionar en estos agradecimientos, porque se lo merecen. Santi, Luis y Amparo, de recepción, por su amabilidad en el trato y su simpatía; Juan Carlos, el guarda que teníamos por las noches, por sus conversaciones a la salida del centro; Pablo Lemos, de informática, por su ayuda en momentos clave, especialmente con las fotos del Capítulo 2.1 de esta Tesis; Niceto y Emilio, de mantenimiento, por intentar siempre arreglar todos los problemas que ha dado el P3, que han sido muchos; el equipo de los invernaderos, en especial Amparo, José Cotanda y Ramón, por su simpatía

y buen trato, y Mª José, Sonia y Cali, por todos mis almuerzos a deshora (es decir, a su hora de almorzar) en el bar.

A todos ellos les agradezco este largo período de convivencia diaria.

En otros laboratorios, tengo que agradecer especialmente a José Luis Palomo toda su ayuda, que ha sido mucha, las muestras de agua del río Tormes con las que se han llevado a cabo las investigaciones que constituyen tres de los cuatro capítulos de esta Tesis, los datos y las fotos que ha aportado para la introducción también de esta Tesis y, sobre todo, su eficacia, su buen hacer y su trato cordial. También a Miguel Cambra y a Ana Palacio, por su simpatía y sus ánimos, y especialmente a Ana porque en su paso por Bacteriología fue muy buena compañera, una "curranta" impresionante y a la vez muy modesta, y luego en la lejanía ha sabido estar ahí, dispuesta a ayudarme en momentos de dificultad. Por supuesto, a Montse Roselló, por todos sus ánimos para acabar con la tesis cuando ella también tenía lo suyo con la suya, y por su trato siempre tan cariñoso.

Je voudrais remercier aux personnes qui, pendant mon stage de presqu' un an à l'INRA de Toulouse, ont parvenu à me faire sentir comme si j'étais chez moi. J'ai de très bons souvenirs de cette époque.

En premier lieu, à André et Dany, ce couple adorable qui m'a ouvert les portes de son labo, qui m'a beaucoup aidé et très bien conseillé et surtout qui m'ont toujours montré une gentillese hors du commun. D'une façon très spéciale, à Jacques Vasse (Jacko) parce que, pendant mon séjour, il a été comme un deuxième père pour moi. Il m'a appris beaucoup de choses sur la culture française, lui aussi il aimait la culture et la langue espagnoles, on a passé beaucoup de temps ensemble au labo et à la cantine et il a été un bon copain de laboratoire, un peu râleur, mais formidable. Je lui remercie aussi de tout ce qu'il m'a appris sur les tecniques microscopiques et l'anatomie végétale. Également merci à Vincent, avec qui j'ai partagé de nombreuses aventures et blagues au labo et en dehors. Tous le trois, nous étions l'équipe des bras cassés, la meilleure des équipes, je pense.

Je voudrais remercier, bien sûr, à l'autre équipe de recherche qui était physiquement dans le même labo que nous. À Matthieu Arlat, qui est vraiement charmant, et qui nous a très souvent fait de blagues aussi

ingénieuses que lui-même; à Claudine et à Manue, qui m'ont beaucoup aidé et se sont toujours montrées très amicales avec moi ; à Damien, avec qui j'ai beaucoup ri et joué au labo ; à Xu et à Cathy, qui, pendant ses séjours respectifs, ont été toujours très sympas avec moi.

Je remercie aux personnes du labo d'en face (La Cytologie), à Françoise, à Mari Christine et à Tim qui ont été si aimables et cordiales avec moi depuis le début et qui m'ont aidé avec de certains appareils.

Merci aussi à des personnes appartenant à d'autres labos et endroits à l'INRA, qui m'ont très bien accuielli et donc qui ont contribué à faire mon stage beaucoup plus agréable : Yves Marco, Jocelyne, Laurent, Susana, Maurice, Pierre Boistard, Christian Boucher, Stéphane Genin, Marc Valls, Karine, Cyril Bontemp, Fabienne Vailleau, Daniel Kahn, Marcella, et les équipes de la serre et la lavérie. Aussi je remercie à Lay, à Marcus et à Carlos, très sympas et avec qui j'ai partagé pas mal de temps en dehors l'INRA. Je m'excuse si j'oublie quelqu'un.

Quiero agradecer a los Evaluadores Externos de esta Tesis y a los Miembros del Tribunal que hayan aceptado estas funciones, sobre todo porque han tenido que leerse esta Tesis en los meses de julio y agosto y, por lo tanto, quitando tiempo de sus vacaciones de verano.

A las Secretarias del Departamento de Biotecnología de la Universidad Politécnica, especialmente a María Jesús Navarro, por facilitar al máximo, a pesar de todos los problemas surgidos en la tramitación de la Tesis, que la lectura tenga lugar a principios de septiembre.

Tengo también mucho que agradecer a mis amigos, Almudena, Mª Eugenia, Puri y Javi, Amparo y José, Olga, Ester Burdeos, Paqui y Eva-Amparo, que no han dejado de darme ánimos y, sobre todo, que no han dejado de ser buenos amigos a pesar de muchas ausencias. A mis amigos en la distancia, Borja y Luis, también Ahmed, a antiguos compañeros de piso que son ya amigos, Ana Belén Ruiz y Daryl, Bea, Óscar, Soraya, Tere y Andrés, que han vivido más de cerca el día a día de mi Tesis y han sabido comprenderme y apoyarme. A amigos más recientes, como Ana y Fernando, que también han tenido buenas palabras para mí.

Por supuesto, a mi familia, padres y hermana, que no entienden muy bien dónde me he metido, pero que están deseando que salga.

Gracias también a este sentido del humor que tengo, y que ha sido mi propia estrategia de supervivencia en estos largos años de Tesis.

CONTENTS

Summary

The *Ralstonia solanacearum* species complex causes bacterial wilt, a plant disease affecting economically important crops and ornamentals worldwide. The phylotype (ph) II race 3 biovar (bv) 2 produces potato brown rot and bacterial wilt in solanaceous plants in temperate climates and has recently been introduced to several areas of the European Union and the USA, where the pathogen has a quarantine status. Presence of *R. solanacearum* ph II race 3 bv 2 in these zones raised questions on biological and phytopathological aspects of this bacterium, some of them being addressed in this work. Thus behaviour, ability for survival and disease inducing capacity of European *R. solanacearum* ph II race 3 bv 2 have been assessed in a range of different plant species and diverse surface run-off water samples.

Behaviour of *R. solanacearum* ph II race 3 bv 2 *in planta* has led to a classification in susceptible or tolerant hosts and non-hosts, based on the pathogen histological localization and isolation. Susceptible and tolerant hosts were highly invaded in root xylem but, heavily or weakly colonized respectively in stem xylem. They are to be avoided as candidates for crop rotation. Non-hosts were not invaded in plant xylem but, occasional presence of the pathogen in root cortex or on surface might occur and so, some of them might act as reservoirs. They could be selected for crop rotation systems after being carefully tested in open field conditions.

Ability for survival of *R. solanacearum* ph II race 3 bv 2 in environmental water microcosms has been influenced by abiotic and biotic factors. When faced to oligotrophy as the only stressing factor the pathogen displayed a considerable endurance. It resorted to a number of survival strategies which enabled it to survive in environmental water microcosms over four years under starvation, retaining disease inducing capacity in the host even by watering. Adaptations to overcome nutrient limitation during the long period were starvation-survival responses, the entrance into a viable but non-culturable state, progressive transformation from the typical bacillar shape into coccoid forms reduced in size, filamentation and budding phenomena and aggregation.

Survival strategies were also successfully exhibited by the pathogen when exposed to environmental temperatures simultaneously to nutrient scarcity conditions. Thus, at 4°C a viable but non-culturable state dependent on water nutrient contents was cold-induced, whilst at 14°C and 24°C apparently similar starvation-survival responses revealed a distinct effect of temperature on coccoid formation.

On the other hand, indigenous freshwater protozoa, bacteria and/or phages with predatory, competitive or lytic activity reduced significantly pathogen persistence. Among them, lytic bacteriophages were the main responsible for the decrease in *R. solanacearum* populations, although protozoa and other bacteria also contributed. The effect was more appreciable at 24°C than at 14°C because of slower biotic interactions at the lower temperature. The pathogen was able to adapt itself, succeeding in surviving and keeping pathogenic in almost all condition.

This work intends to contribute to the progress in the knowledge of the interactions between *R. solanacearum* ph II race 3 bv 2 and natural environments, which may allow to improve the strategies to prevent pathogen dissemination and bacterial wilt spread in natural settings.

Resumen

El complejo de especies de *Ralstonia solanacearum* es el responsable de la marchitez bacteriana, una enfermedad que afecta en todo el mundo a cultivos y plantas ornamentales de gran importancia económica. El filotipo (ft) II raza 3 biovar (bv) 2 produce la podredumbre parda de la patata y la marchitez bacteriana de las solanáceas en climas templados, y se ha introducido recientemente en algunas áreas de la Unión Europea y de Estados Unidos, donde este patógeno se considera un organismo de cuarentena. La presencia de *R. solanacearum* ft II raza 3 bv 2 en estas zonas ha hecho surgir ciertos interrogantes sobre la biología y la patogenicidad de esta bacteria, algunos de los cuales se han abordado en este trabajo. De esta manera, se han evaluado aspectos del comportamiento, la capacidad de supervivencia y la capacidad para la inducción de la enfermedad, de cepas de *R. solanacearum* ft II raza 3 bv 2 de origen europeo en diferentes especies vegetales y diversas muestras de agua medioambiental.

El comportamiento en cuanto a la colonización de *R. solanacearum* ft II raza 3 bv 2 *in planta* ha permitido realizar una clasificación en huéspedes susceptibles o tolerantes y en no huéspedes, en base a la localización histológica del patógeno y su aislamiento. Se ha observado que los huéspedes susceptibles y tolerantes eran intensamente invadidos a nivel del xilema de las raíces, pero densamente o bien débilmente colonizados, respectivamente, a nivel del xilema del tallo. Ninguno de ellos debe ser considerado como candidato para la rotación de cultivos en suelos contaminados. Por otra parte, se ha observado que en los no huéspedes no se producía la invasión a nivel del xilema de la planta, aunque podría haber presencia esporádica del patógeno en el córtex de las raíces o en la superficie y, por lo tanto, algunos podrían actuar como reservorios. Los no huéspedes podrían seleccionarse para los sistemas de rotación tras su evaluación en condiciones de campo.

La capacidad de supervivencia de *R. solanacearum* ft II raza 3 bv 2 en microcosmos de agua medioambiental se ha visto influida por factores tanto abióticos como bióticos. El patógeno ha mostrado una resistencia considerable frente a la exposición a la oligotrofía como único factor de stress. Así, desarrolló varias estrategias que le permitieron sobrevivir en

microcosmos de agua medioambiental durante cuatro años bajo condiciones de limitación de nutrientes, manteniendo la capacidad de inducir la enfermedad en el huésped incluso a través de riego. Las estrategias adaptativas para superar la oligotrofía observadas durante este largo periodo han sido la respuesta de supervivencia frente a la privación de nutrientes, la entrada en el estado viable no cultivable, una transformación progresiva de las formas bacilares típicas en cocoides de reducido tamaño, los fenómenos de filamentación y gemación, y la agregación.

El patógeno también ha mostrado estrategias eficientes cuando ha sido expuesto a diferentes temperaturas simultaneamente a las condiciones de escasez de nutrientes. De esta manera, a 4°C el frío indujo la entrada en un estado viable no cultivable que fue dependiente del contenido en nutrientes del agua, mientras que a 14°C y 24°C las respuestas de supervivencia frente a la privación de nutrientes, aunque aparentemente similares, revelaron un efecto de la temperatura en la formación de los cocoides.

Por otra parte, la persistencia del patógeno se redujo de manera significativa en microcosmos de agua medioambiental por la actividad predadora, competitiva o lítica de los protozoos, las bacterias y/o los bacteriófagos presentes en el agua. Entre todos ellos, se demostró que los fagos líticos fueron los responsables principales del descenso en las poblaciones de *R. solanacearum*, si bien los protozoos y las otras bacterias contribuyeron en cierta medida. El efecto fue más apreciable a 24°C que a 14°C debido a que las interacciones bióticas fueron más lentas a la temperatura más baja. Esta bacteria ha mostrado una gran capacidad de adaptación, consiguiendo sobrevivir y mantener su poder patógeno en prácticamente todas las condiciones ensayadas.

La finalidad de este trabajo ha sido contribuir al progreso del conocimiento de las interacciones entre *R. solanacearum* ft II raza 3 bv 2 y sus ambientes naturales, para mejorar las estrategias preventivas tanto de la diseminación del patógeno como de la propagación de la marchitez bacteriana en dichos hábitats.

Résumé

Le complexe d'espèces de *Ralstonia solanacearum* est l'agent causal du flétrissement bactérien, une maladie qui affecte des cultures et des plantes ornementales de grande importance économique dans le monde entier. Le phylotype (phyl) II race 3 biovar (bv) 2 produit la pourriture brune de la pomme de terre et d'une manière générale le flétrissement bactérien des Solanacées dans les climats tempérés. Ce phylotype II a récemment été introduit dans des certaines régions de l'Union Européenne et des États-Unis, où cette bactérie est considérée comme un pathogène de quarantaine. La présence de *R. solanacearum* phyl II race 3 bv 2 dans ces régions a soulevé quelques questions au sujet de sa biologie et de son pouvoir pathogène, questions qui ont été abordées dans ce travail. Nous avons notamment évalué, chez *R. solanacearum* phyl II race 3 bv 2 d'origine européenne, l'aptitude à survivre dans divers échantillons de l'eau environnementale de surface et la capacité à induire la maladie dans des différentes espèces de plantes cultivées.

Notre étude de la capacité de colonisation de *R. solanacearum* phyl II race 3 bv 2 *in planta* a permis une classification en hôtes sensibles ou tolérants et non hôtes sur la base de la localisation histologique de l'agent pathogène et de son isolement *in planta*. Il a été noté que les hôtes sensibles et tolérants sont intensément envahis à niveau du xylème des racines, mais fortement ou faiblement colonisés à niveau du xylème de la tige chez les plantes sensibles ou tolérantes respectivement. Aucune de ces plantes ne doit être recommandée comme candidat dans une rotation culturale. En outre, nous avons noté que le xylème des plantes non hôtes n'est pas envahi, bien que l'on puisse noter la présence sporadique de l'agent pathogène dans le cortex des racines ou sur la surface racinaire. Cela signifie que certaines plantes non hôtes pourraient potentiellement être des réservoirs d'inoculum latent. En conséquence les plantes non hôtes qui pourraient être recommandées dans des systèmes de rotation culturale doivent être évaluées non seulement au laboratoire mais aussi au champ dans des conditions naturelles.

Nous avons montré que la capacité de survie de *R. solanacearum* phyl II race 3 bv 2 dans des microcosmes de l'eau environnementale,

préalablement filtrée ou autoclavée, est influencée par des facteurs abiotiques et biotiques. L'agent pathogène fait preuve d'une résistance considérable quand il est exposé à l'oligotrophie comme seul facteur de stress. Nous avons mis en évidence différentes stratégies utilisées par cette bactérie pour survivre plus de quatre ans dans des conditions de limitation des éléments nutritifs dans des microcosmes de l'eau environnementale tout en maintenant sa capacité d'induction de la maladie chez un hôte sensible à la suite de sa dissémination par irrigation. Les adaptations utilisées par cette bactérie pour surmonter l'oligotrophie au cours de cette longue période ont été l'entrée dans l'état viable non cultivable, une transformation progressive des typiques formes bacillaires vers des formes coccoïdes de petite taille, des phénomènes de filamentation et de bourgeonnement et d'agrégation.

L'agent pathogène a également utilisé des stratégies efficaces quand il a été exposé à des différentes températures de l'environnement en même temps qu'à des conditions de limitation des éléments nutritifs. Ainsi à 4°C le froid a entraîné un état viable non cultivable qui a été dépendant de la teneur en éléments nutritifs de l'eau. Par contre à 14°C et 24°C les réponses de survie face à la privation des éléments nutritifs ont induit la formation de formes coccoïdes.

En outre, la persistance de l'agent pathogène a été considérablement réduite dans des microcosmes de l'eau environnementale par l'activité prédatrice, compétitive ou lytique des protozoaires, des bactéries et/ou des bactériophages qui étaient contenus dans de l'eau. Notamment, il a été constaté que les phages lytiques sont les principaux responsables de la baisse des populations de *R. solanacearum*, bien que les protozoaires et d'autres bactéries aient également contribués dans une certaine mesure. L'effet a été plus sensible à 24°C qu'à 14°C en raison d'interactions biotiques plus lentes aux températures plus basses. En conclusion cette bactérie a démontré une grande capacité pour s'adapter, survivre et de maintenir son pouvoir pathogène dans pratiquement toutes les conditions couramment rencontrées dans les conditions naturelles.

Le but de ce travail a été de contribuer à l'avancement de la connaissance des interactions entre *R. solanacearum* phyl II race 3 bv 2 et les

milieux aquatiques naturels ou les plantes hôtes potentielles, dans l'espoir de développer les meilleures stratégies pour empêcher la propagation de l'agent pathogène et donc du flétrissement bactérien dans ces environnements.

Resum

El complex d'espècies de *Ralstonia solanacearum* és l'agent causal del pansiment bacterià, una malaltia vegetal que afecta a tot el món a cultius i plantes ornamentals de gran importància econòmica. El filotipo (fil) II raça 3 biovar (bv) 2 produeix la podridura marró de la patata i el pansiment bacterià en plantes solanàcies de climes temperats, i s'ha introduït recentment en algunes àrees de la Unió Europea i dels Estats Units, on aquest patogen es considera de quarantena. La presència de *R. solanacearum* fil II raça 3 bv 2 en aquestes zones ha fet sorgir certs interrogants sobre qüestions biològiques i fitopatològiques d'aquest bacteri, algunes de les quals s'han abordat en aquest treball. D'aquesta manera s'han valorat aspectes del comportament, la capacitat de supervivència i la capacitat per a la inducció de la malaltia, de *R. solanacearum* fil II raça 3 bv 2 de procedència europea en diferents espècies vegetals i diverses mostres d'aigua d'escorrentia superficial.

El comportament pel que fa a la colonització de *R. solanacearum* fil II raça 3 bv 2 *in planta* ha proporcionat una classificació en hostes susceptibles o tolerants i en no hostes, en base a la localització histològica del patogen i el seu aïllament. S'observà que els hostes susceptibles i tolerants eren intensament envaïts a nivell del xilema de les arrels, però densament o bé feblement colonitzats respectivament a nivell del xilema de la tija. Cap d'ells ha de ser considerat com a candidat per a la rotació cultural. D'altra banda, es va observar que els no hostes no eren envaïts en el xilema de la planta, encara que podria haver presència esporàdica del patogen en el còrtex de les arrels o a la surperfície i, per tant, alguns podrien actuar com a reservoris. Els no hostes podrien ser seleccionats per als sistemes de rotació cultural després d'haver estat avaluats en condicions de camp.

La capacitat de supervivència de *R. solanacearum* fil II raça 3 bv 2 en microcosmos d'aigua mediambiental s'ha vist influïda per factors abiòtics i biòtics. El patogen ha mostrat una resistència considerable quan ha estat exposat a l'oligotrofia com a únic factor d'estrès. Així, va recórrer a diverses estratègies que li van permetre sobreviure en microcosmos d'aigua mediambiental durant quatre anys sota condicions de limitació de nutrients, mantenint la capacitat per a la inducció de la malaltia en l'hoste

fins i tot a través de reg. Les adaptacions per superar l'oligotrofia que s'han observat durant aquest llarg període han estat respostes de supervivència front a la privació de nutrients, l'entrada en l'estat viable no cultivable, una transformació progressiva de les típiques formes bacilars a cocoids de reduït tamany, els fenòmens de filamentació i gemmació, i l'agregació.

El patogen també ha mostrat estratègies eficients quan ha estat exposat a diferents temperatures mediambientals simultàneament a les condicions d'escassetat de nutrients. D'aquesta manera, a 4°C el fred va induir un estat viable no cultivable que va ser dependent del contingut en nutrients de l'aigua, mentre que a 14°C i 24°C les respostes de supervivència front a la privació de nutrients, encara que aparentment similars, van revelar un efecte de la temperatura a la formació dels cocoids.

D'altra banda, la persistència del patogen s'ha vist significativament reduïda en microcosmos d'aigua mediambiental per l'activitat predadora, competitiva o lítica dels protozous, els bacteris i/o els bacteriòfags que contenia l'aigua. Entre tots ells, es va determinar que els fags lítics eren els responsables principals del descens en les poblacions de *R. solanacearum*, si bé els protozous i els altres bacteris van contribuir en certa mesura. L'efecte va ser més apreciable a 24°C que a 14°C degut a que les interaccions biòtiques van ser més lentes a la temperatura més baixa. Aquest bacteri ha mostrat una gran capacitat d'adaptació, aconseguint sobreviure i mantenir el seu poder patogènic en pràcticament totes les condicions.

La finalitat d'aquest treball és contribuir al progrés del coneixement de les interaccions entre *R. solanacearum* fil II raça 3 bv 2 i els seus ambients naturals, el que pot desenvolupar una millora de les estratègies per a la prevenció tant de la disseminació del patogen com de la propagació del pansiment bacterià en aquests ambients.

ABBREVIATIONS

Avr	Avirulence
BDB	Blood Disease Bacterium
bv	biovar
CABI	Commonwealth Agricultural Bureaux International
Cbh	Cellobiohydrolase
cfu	Colony forming units
cv	Cultivar
Da	Daltons
DASI-ELISA	Double-Antibody-Sandwich Indirect Enzyme-Linked Immunosorbent Assay
dpi	Days post-inoculation
DNA/RNA	Deoxyribo/Ribonucleic Acid
Egl	Endoglucanase
EPPO	European Plant Protection Organization
EPS	Exopolysaccharide
GC	Guanine Citosine
HDF	HrpB-Dependent Factor
HR	Hypersensitive Response
Hrp	Hypersensitive reaction & pathogenicity
ITS	Intergenic Spacer
kb	Kilobases
LPS	Lipopolysaccharide
Mb	Megabases
MNP	Most Probable Number
ORF	Open Reading Frame
PC/ Phc	Phenotype Conversion
PCR	Polymerase Chain Reaction
ph	phylotype
Peh	Polygalacturonase
Pme	Pectin methyl esterase
RFLP	Restriction Fragment Length Polymorphism
SMSA	Semiselective Medium South Africa
T2SS / T3SS	Type 2/ Type 3 Secretion System
VBNC	Viable But Non-Culturable
YPGA	Yeast Peptone Glucose Agar
3-OH PAME	3-hydroxypalmitic acid methyl ester

Problem statement & Research objectives

In the last fifteen years *Ralstonia solanacearum* phylotype (ph) II race 3 biovar (bv) 2 has been introduced to several countries of the European Union (EU), where the pathogen is considered a quarantine organism. The presence of the bacterium in these areas has been related to outbreaks of bacterial wilt and/or potato brown in solanaceous crops. In spite of the efficiency of the eradication measures applied by EU Directives whenever a new focus is detected, the disease still poses a threat to crops and ornamentals in European countries, included Spain.

Up to the accomplishment of this research work, scarce information was available on several biological and phytopathogenic characteristics of the *R. solanacearum* species complex, especially with respect to the ph II race 3 bv 2, which is considered the European ph. These characteristics would mainly refer to the role that the natural environments can play in terms of affecting the pathogen behaviour, its ability for survival and its disease inducing capacity. Their study might provide with valid knowledge to develop new strategies and/or improve those already existing to prevent and/or control the bacterial wilt disease.

Consequently, a general objective of this research work has been to address some of the interactions of the European ph of *R. solanacearum* with common host and non-host environments, which should be investigated to improve the knowledge about the life cycle of *R. solanacearum* ph II and the epidemiology of the disease in different habitats.

This main research objective has been divided into the following working objectives proposed:

1- To establish a classification system of plant species permitting a clear cut distinction between hosts and non-hosts for *R. solanacearum*.

2- To find potential crop candidates for rotation schemes in *R. solanacearum* ph II race 3 bv 2-threatened areas, to be further evaluated in open field conditions to confirm their suitability.

3- To ascertain whether *R. solanacearum* ph II race 3 bv 2 is able to survive in natural water and retain pathogenicity when faced to prevailing environmental abiotic factors such as:

3.1 oligotrophy under extended periods of time
3.2 oligotrophy at various temperature conditions.

4- To assess the influence of environmental biotic factors such as different fractions of natural water microbiota on the population dynamics of *R. solanacearum* ph II race 3 bv 2 and the effect of temperature on those interactions.

The first and second working objectives, the plant classification system and the crop rotation candidates, are described in the Chapter 2.1, and the findings have been published in *Phytopathology* (2008) 98: 59-68.

The results of the third working objective, the effect of abiotic factors on the pathogen, are reported throughout the Chapters 2.2 and 2.3:

- The effect of long-term oligotrophy (objective 3.1) has been developed in the Chapter 2.2 and has led to a publication in *Microbiology* (2008) 154: 3590–3598.

- The simultaneous effect of temperature and oligotrophic conditions (objective 3.2) has been investigated and described in the Chapter 2.3 and the knowledge provided has been summarized in a manuscript to be submitted for publication in 2009.

Finally, the work related to the fourth working objective, the effect of biotic factors on the pathogen, is reported in the Chapter 2.4, whose contributions have been published in *Applied and Environmental Microbiology* (2007) 73 (22): 7210–7217.

PART 1
INTRODUCTION

GENERAL ASPECTS OF THE BACTERIAL WILT DISEASE AND ITS CAUSATIVE AGENT, THE *Ralstonia solanacearum* SPECIES COMPLEX

1.1 The bacterial wilt disease

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most important and widespread bacterial diseases of plants. The origin and subsequent dissemination of the disease remain undetermined. The earliest reports were published about the same time, towards the end of the 19th century, in diverse parts of Asia, South America, USA and Australia, where it was already apparently well-established (Kelman, 1953). Within this period, intensification of the culture of the more susceptible solanaceous hosts such as tomato, potato and tobacco in subtropical or warm-temperate zones made the disease easier to be fully recognized (Kelman, 1953). This destructive parenchymo-vascular wilt disease was first described by E.F. Smith from potato, tomato and eggplant in 1896 (Smith, 1896) and subsequently from tobacco in 1908 (Smith, 1908). Since then, it has been reported on a great many plant species belonging to a wide range of plant families, keeping a broad geographical distribution, and producing severe economic losses worldwide.

1.1.1 Symptomatology

Bacterial wilt most characteristic external symptoms are wilting, stunting and yellowing of the foliage (Smith, 1920; Kelman, 1953) (Fig. 1.1). Frequently leaves are bent downward showing leaf epinasty (Smith, 1914, 1920). Adventitious roots appear in the stems, and narrow dark stripes corresponding to the infected vascular bundles may be observed beneath the epidermis (Smith, 1914, 1920; Kelman, 1953). Symptoms may appear at any stage in the growth of the susceptible host, although complete wilting and collapse are more frequent in younger infected plants (Smith, 1914; Kelman, 1953). Even though typical wilt symptoms may fail to develop, dwarfing and stunting of plants may occur (Smith, 1914; Kelman, 1953). Expression of the symptoms and rate of disease development may vary according to the susceptibility and growth conditions of the host plants, and would also be influenced by environmental conditions (Smith, 1914; Kelman, 1953).

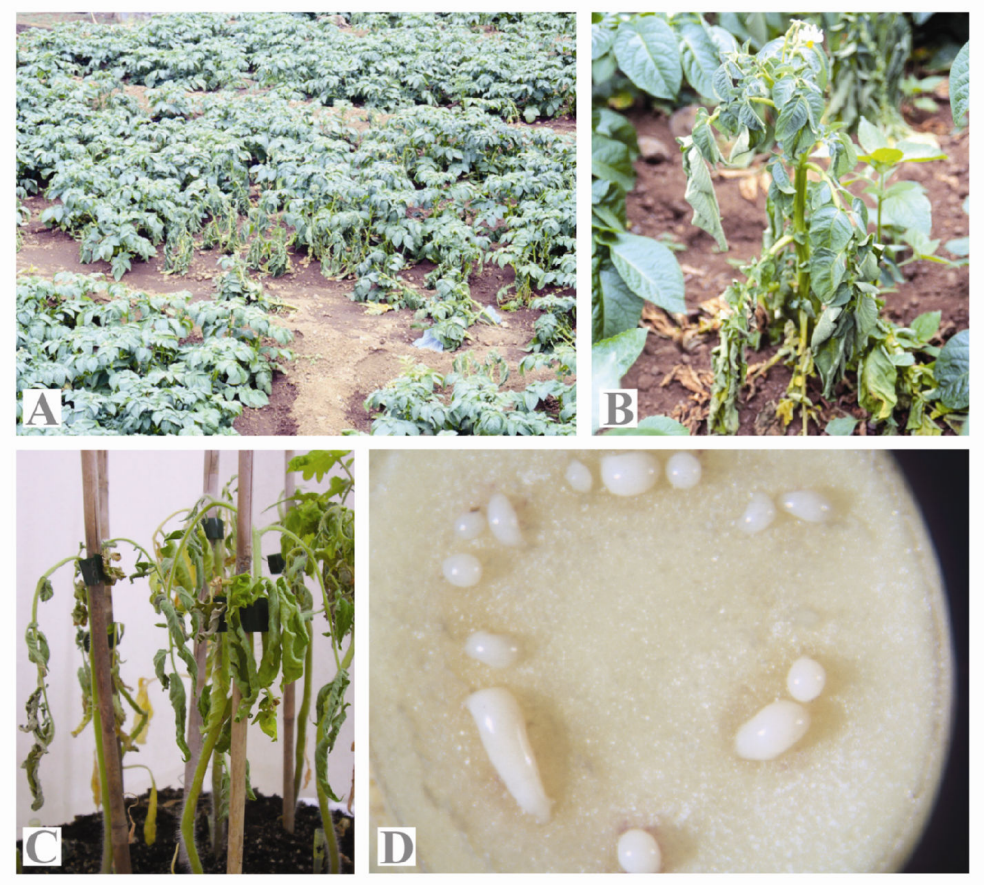


Figure 1.1. Wilting symptoms in potato plants (A and B) and tomato plants (C), and brown rot in a tuber (D). [(A and B) F. Siverio/M.M. López, (C) B. Álvarez, (D) A. Trigalet].

Internal symptoms can be observed after examination of sectioned diseased plant tissues, and usually reveal yellowish to brownish discoloration of the vascular tissue at early stages of infection, and portions of the pith and cortex becoming deep brown as they are invaded and destroyed (Kelman, 1953). The stem may be honey-combed for long distances with bacterial cavities (Smith, 1920). Longitudinal sections of stems may show narrow dark brown to black lines in the xylem and the basal part often becomes hollow due to pith decay and the necrosis of vascular tissue (Kelman, 1953). A typical sign of disease is the appearance of slimy viscous ooze on a transverse-sectioned stem at the points

corresponding to the vascular bundles (Smith, 1896). A plant with the first foliar symptoms of bacterial wilt will have relatively few roots and often only one in an advanced state of decay (Kelman, 1953). As disease develops in the plant, most of them progressively wilt, becoming dark-brown to black (Kelman, 1953). Degradation of the occluded xylem vessels combined with extensive invasion and destruction of surrounding tissues (phloem, cortex and pith) produce collapse and death of the plant (Smith, 1920; Kelman, 1953).

In potato tubers, vascular infection by *R. solanacearum* causes the potato brown rot (Smith, 1896, 1920; Kelman, 1953) (Fig. 1.1). Transverse sections of diseased tubers frequently show brown discoloration and decay localized in the vascular ring, with grey bacterial slime oozing at certain points to the surface (Smith, 1920). When the disease is advancing, these stained tubers may show on their surfaces discolorated, darkened areas while keeping their skin unbroken and outer tissues sound (Smith, 1920; Kelman, 1953). The diseased tuber is eventually completely destroyed.

1.1.2 Host range

R. solanacearum has a large and expanding host range comprising more than 200 plant species belonging to 50 botanical families, the most frequently affected species being from *Solanaceae* and *Musaceae* (Kelman, 1953; Buddenhagen & Kelman, 1964; Hayward, 1964, 1994; Elphinstone, 2005).

The most important hosts worldwide are: banana and plantain (*Musa paradisiaca*), eggplant (*Solanum melongena*), groundnut (*Arachis hypogaea*), *Heliconia* spp., potato *(S. tuberosum*), tobacco *(Nicotiana tabacum*), and tomato (*Lycopersicon esculentum*) (EPPO, 2004). Major economic losses have been produced in tomato, potato, tobacco, banana, groundnut, and ginger (*Zingiber officinale*) infected crop fields (Elphinstone, 2005).

Based on host range, *R. solanacearum* strains have traditionally been classified into races, with five of them designated so far (Buddenhagen *et al*., 1962; Buddenhagen & Kelman, 1964; Hayward, 1991). According to Elphinstone (2005), the most frequently reported hosts for each of the different pathogenic *R. solanacearum* races in the last years have been:

For race 1, solanaceous crops like chili and sweet pepper (*Capsicum annuum*), eggplant, potato, tobacco and tomato; non-solanaceous crops like bean (*Phaseolus vulgaris*), groundnut and sunflower (*Helianthus annuus*); ornamental plants like *Anthurium* spp., *Dahlia* spp., *Heliconia* spp., *Hibiscus* spp., *Lesianthus* spp., *Lilium* spp., marigold (*Tagetes* spp.), palms, *Pothos* spp., *Strelitzia* spp., *Verbena* spp. and *Zinnia* spp.; trees like *Eucalyptus* and fruit trees as black sapote (*Diospyros digyna*), custard apple (*Annona squamosa*), and neem (*Azadirachta indica*). Also, although of minor importance: abaca (*Musa textilis*), cowpea (*Vigna sinensis*), cucurbits, hyacinth beans (*Dolichos lablab*), jute (*Corchorus* spp.), moringa (*Moringa olerifera*), mulberry (*Morus* spp.), nutmeg (*Myristica* spp.), patchouli (*Pogostemon patchouli*), *Perilla crispa*, sesame (*Sesamum indicum*), strawberry (*Fragaria* spp.), water spinach (*Ipomoea reptans*), wax apple (*Syzygium samarangese*) and winged bean (*Psophocarpus tetragonolobus*).

For race 2, cooking and dessert bananas, plantain, other *Musa* spp. and wild and ornamental *Heliconia* spp.

For race 3, *Capsicum* spp., eggplant, geraniums (*Pelargonium* spp.), potato and tomato; weeds like *Solanum dulcamara* and *S. nigrum*.

For race 4, ginger and the related plant species mioga (*Zingiber mioga*) and patumma (*Curcuma alismatifolia*).

For race 5, *Morus* spp.

1.1.3 Geographical distribution

The worldwide distribution of zones where the bacterial wilt disease has been found and confirmed is periodically summarized by the Commonwealth Agricultural Bureaux International (CABI) and the European and Mediterranean Plant Protection Organisation (EPPO) (Elphinstone, 2005), which distribute updated maps of the disease for the three races with a wider spread (Fig. 1.2). According to the most recently available maps (EPPO/CABI, 2006), *R. solanacearum* race 1 is present in the five continents, included Europe, with the exception of the European Union (EU) member states (Fig. 1.2 above); race 2 occurs mainly in tropical areas of South America and also in the Philippines (Fig. 1.2 middle) and race 3 is widespread in all the five continents (Fig. 1.2 below).

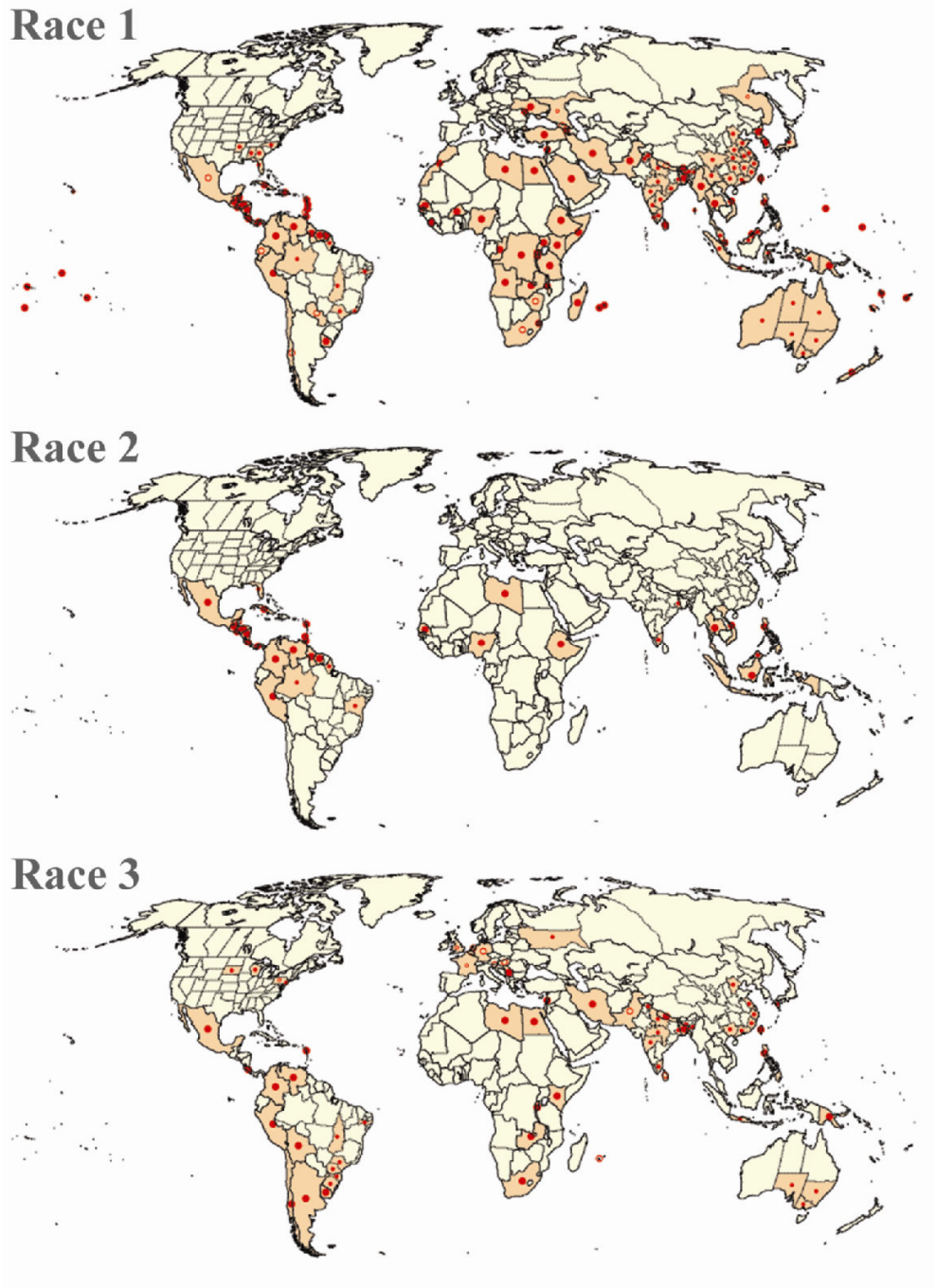


Figure 1.2. Worldwide distribution of *R. solanacearum* races 1, 2 and 3. [From EPPO/CABI (2006), for the period 2003-06].

Races 4 and 5 occur in Asia, with race 5 being limited to China (Elphinstone, 2005). Africa is the continent where the highest diversity of this pathogen can be found (Elphinstone, 2005).

In the EU, *R. solanacearum* race 3 is present in several countries and has potential for spread (EPPO, 2004). Outbreaks of potato brown rot caused by race 3 were firstly reported in Sweden in 1976 (Olsson, 1976), and have been reported since 1989 in restricted areas of EU countries such as Belgium, France (De Guenin, 1998), Germany, Greece, Hungary, Italy (Turco, 1995; Calzolari *et al.*, 1998), the Netherlands (Janse, 1996; Janse & Schans, 1998; Janse *et al.*, 1998), Portugal, Spain (Palomo *et al.*, 2000; Caruso *et al.*, 2005), Sweden (Persson, 1998) and the United Kingdom (Elphinstone *et al.*, 1998a, b). Since no clonal linkage was established among the causative strains, it seems that the outbreaks were independent and so a genetically distinct "European" strain of *R. solanacearum* does not exist (Hayward *et al.*, 1998). In some cases the infestation routes are not exactly known, but the first outbreaks seem to be related to latent infections in imported potatoes coming from tropical or subtropical regions where the disease is endemic (Olsson, 1976; Janse *et al.*, 1998). Global climate change may have been also a factor influencing the introduction of the pathogen in the EU, since several warm summers occurred in Western Europe in the early 1990's (Hayward *et al.*, 1998). Subsequent dissemination would have been related to the introduction and/or increase of irrigation to these areas (Olsson, 1976; Hayward *et al.*, 1998), suggesting that the pathogen might have adapted to European soils and surface run-off waters. Although strict control measures imposed through official EU Directives (Anonymous, 1998, 2006) have significantly reduced the risk of *R. solanacearum* dissemination, eradication has not been so far successfully achieved (Elphinstone, 2005). Furthermore, there is currently a risk of introduction of *R. solanacearum* race 1 with ornamental/herbal plants and plant parts from tropical areas that can grow in glasshouses in temperate climates, like *Curcuma longa* (turmeric), *Anthurium* or *Epipremnum* (EPPO, 2004).

1.1.4 *R. solanacearum* in Spain

In Spain, the two earliest reports on bacterial wilt date back to 1928 (Anonymous, 1928) near Barcelona, and 1945 (Sardiña, 1945) in Galicia, both from potato (Kelman, 1953).

More recently, in 1992, the pathogen was sporadically detected in the Islas Canarias (EPPO/CABI, 1992), and four years later, in 1996, it was anew found in the Islas Canarias (La Palma) and also in Galicia (La Coruña) from seed potato (Janse, 1996) (Fig. 1.3). Surveys performed from 1999 to 2008 succedded in finding the pathogen in several regions of Spain (Fig. 1.3): in Castilla y León, outbreaks have been detected in Burgos, León, Palencia, Salamanca, Segovia, Soria, Valladolid and Zamora from potato, seed potato and surface waters; in País Vasco, in Álava from potato; in Extremadura, in Badajoz and Cáceres from tomato and surface waters; and in Andalucía, in Almería and Granada from tomato (Palomo *et al.*, 2000, 2002, 2004; Caruso *et al.*, 2005) (Fig. 1.3). In all cases, eradication measures were applied (Anonymous 1998, 2006).

Figure 1.3. Detections of *R. solanacearum* in Spain in the last fifteen years.

1.1.5 Economic importance

The economic impact of the disease is known to be enormous worldwide but, it is difficult to accurately estimate the losses due to an undocumentable effect on subsistence agriculture and the abandonment of highly susceptible crops in many parts of the world (Hayward, 1991; Elphinstone, 2005).

Losses greatly differ in the distinct areas in dependance on (i) local climates, soil types and cropping practices, (ii) the choice of crop and plant cultivar, and (iii) the virulent characteristics of the *R. solanacearum* strains present (Elphinstone, 2005). In zones as the EU, where the pathogen is considered a quarantine organism (Anonymous, 2000), severe economic damage may be also related to the fact that Directives (Anonymous, 1998, 2006) impose destruction of entire infected crops, restriction on further production on infested land, prohibition of the use of contaminated waterways, and additional eradication measures when required (Anonymous, 1998, 2006; Elphinstone, 2005; López & Biosca, 2005).

1.2 *Ralstonia solanacearum*, the causative agent

R. solanacearum (Smith, 1896) Yabuuchi *et al.* (1995) is a bacterial plant pathogen which belongs to the β-proteobacteria. The pathogen comprises a "species complex" provided its high heterogeneity (Fegan & Prior, 2005), and possesses several pathogenicity and virulence determinants, controlled by a complex, density-dependent regulatory network (Schell, 2000). The bacterium is soil and water borne, enters the plant via the roots, moves systemically through the xylem and causes lethal disease (Kelman, 1953). It is able to survive in the environment in the absence of a host, mainly in water, soil, plant debris and reservoir plants.

1.2.1 Taxonomy

Although T.J. Burrill was probably the first to isolate the bacterium approximately in 1890 (Burrill, 1890, 1891; Kelman 1953), E.F. Smith was the first to fully describe it and to demonstrate its pathogenicity on tomato and potato (Smith, 1896). Since it seemed to have peritrichous flagella, it

was classified in the genus *Bacillus* and was assigned to the species name of *B. solanacearum* in 1896 (Smith, 1896; Kelman, 1953). Two years later it was moved to the genus *Bacterium* (Chester, 1898; Smith, 1908), and then in 1914, E.F. Smith suggested to refer to it as *Pseudomonas solanacearum* (Smith, 1914) provided it was found to have a single polar flagellum (Smith, 1914; Kelman, 1953). Subsequently the bacterium was temporary reclassified in the genera *Phytomonas* (Bergey *et al*., 1923) and *Xanthomonas* (Dowson, 1939) on the basis of cultural and biochemical characteristics, and eventually transferred back to the genus *Pseudomonas* in 1948 (Dowson, 1948). Within this genus, *P. solanacearum* was included in the rRNA homology group II, with other non-fluorescent pseudomonads (Palleroni & Doudoroff, 1971). In 1992 most of this group was proposed to be placed in the new genus *Burkholderia* on the grounds of 16S rRNA sequences, DNA-DNA homologies, fatty acid analyses, and other phenotypic characteristics (Yabuuchi *et al*., 1992). More recent phylogenetic and polyphasic phenotypic analyses (Gillis *et al*., 1995) pointed out that *B. solanacearum*, together with *B. pickettii* (former *P. pickettii*) and *Alcaligenes eutrophus*, would rather be accommodated in the new established genus of *Ralstonia*, in 1995 (Yabuuchi *et al*., 1995). Since then, the bacterium is named *Ralstonia solanacearum* and belongs to the family *Ralstoniaceae* included in the β-subdivision of the Proteobacteria (Stackebrandt *et al*., 1988).

Furthermore, in the following years several novel species have been described in the genus *Ralstonia*: *R. basilensis* (Steinle *et al*., 1998; Goris *et al*., 2001), *R. campinensis* (Goris *et al*., 2001), *R. gilardii* (Coenye *et al*., 1999), *R. insidiosa* (Coenye *et al*., 2003a), *R. metallidurans* (Goris *et al*., 2001), *R. mannitolilytica* corrig. (De Baere *et al*., 2001; original spelling *R. mannitolytica*), *R. oxalatica* (Sahin *et al*., 2000), *R. paucula* (Osterhout *et al*., 1998; Vandamme *et al*., 1999; Moissenet *et al*., 1999), *R. respiraculi* (Coenye *et al*., 2003b) and *R. taiwanensis* (Chen *et al*., 2001). The type species of the genus is *R. pickettii* (Yabuuchi *et al*., 1995).

Subsequently, comparative 16S rDNA sequence analysis indicated that two distinct groups existed within the genus *Ralstonia* (Vaneechoutte *et al*., 2004): the *R. eutropha* lineage, comprising *R. basilensis*, *R. campinensis*, *R. eutropha*, *R. gilardii*, *R. metallidurans*, *R. oxalatica*, *R. paucula*, *R. respiraculi* and

R. taiwanensis; and the *R. pickettii* lineage, comprising *R. insidiosa*, *R. mannitolilytica*, *R. pickettii*, *R. solanacearum* and *R. syzygii* (previously *P. syzygii*, renamed based on new DNA–DNA hybridization data; Vaneechoutte *et al.*, 2004). This phylogenetic discrimination was supported by phenotypic differences: members of the *R. eutropha* lineage have peritrichous flagella, do not produce acids from glucose and are susceptible to colistin, whereas members of the *R. pickettii* lineage have one or more polar flagella, produce acid from several carbohydrates and are colistin-resistant. The *R. eutropha* lineage was re-classified in the new genus *Wautersia* in 2004 (Vaneechoutte *et al.*, 2004).

1.2.2 Morphological & physiological characteristics

Shape and size of *R. solanacearum* were first described by Smith (1896). The single cell is a small rod with rounded ends whose length is usually 1.5 to 3 times greater than the width (Smith, 1896; Kelman, 1953); an average size would be 0.5 to 0.7 by 1.5 to 2.5 μm (Denny & Hayward, 2001). Cell wall structure is that of Gram-negative bacteria and flagella when present are polar. *R. solanacearum* produces poly-β-hydroxybutyrate granules as cell energetic reserve, which can be observed by staining with either Sudan black or Nile blue (Anonymous, 1998, 2006). The bacterium has an oxidative metabolism and is generally considered a strict aerobe. However, under some circumstances, it is able to limited, slow growth when cells are not in direct contact with the air (Kelman & Jensen, 1951; Kelman, 1953). *R. solanacearum* physiological and biochemical characteristics have been extensively reviewed (Kelman, 1953; Anonymous, 1998, 2006; Denny & Hayward, 2001; EPPO, 2004). The main distinctive tests for the species are displayed in Table 1.1.

With respect to growth temperature, *R. solanacearum* strains from tropical areas all over the world have a high temperature optimum (35°C), whereas that of strains occurring at higher altitudes in the tropics and in subtropical and temperate areas is lower (27°C); no growth has been observed at 40°C or 4°C (EPPO, 2004). Approximative minimal and maximal growth temperature values would be 8-10°C and 37-39°C respectively (Kelman, 1953). Regarding pH requirements, in general *R.*

solanacearum growth is inhibited in acid media but favoured in alkaline conditions (Kelman, 1953). A valid range of pH permissive for growth can vary depending on the culture media but, it might stand between 4 and 9, being weak at around 8 and optimal between 6 and 7 (Kelman, 1953; EPPO, 2004). *R. solanacearum* can grow in 1% NaCl liquid media but, little or none in 2% NaCl (EPPO, 2004).

Table 1.1. Summary of *R. solanacearum* biochemical characteristics. [Adapted from Denny & Hayward (2001) and EPPO (2004); picture, a *R. solanacearum* cell observed by electronic microscopy, J. Vasse].

Catalase	+
Oxidase	+
Nitrate reduction	+
Gas by denitrification	frequent
Gelatin liquefaction	weak / -
Levan formed from sucrose	-
Esculin hydrolysis	-
Starch hydrolysis	-
Arginine dihydrolase	-
Lecithinase	-
Lipase (Tween 80)	-

- Most strains produce tyrosinase, the main exceptions being those isolated from the family *Musaceae*.
- Total cellular fatty acids have less than 1% of cyclopropanoic acid (C19:0).
- Cellular fatty acids lack ornithine lipids OL-1 and OL-2.

Two morphological types of *R. solanacearum* colonies can be typically observed on agar plates (Fig. 1.4): fluidal or mucoid and afluidal or non-mucoid. Mucoid colonies are rather watery, wet-shining, irregularly round and usually have a tendency to flow when placed in a vertical or semi-vertical position (Smith, 1920). The mucoid substance is produced by the accumulation of an extracellular polysaccharide (EPS), which causes these mucoid colonies to exhibit a typical irregularity of their surfaces

(Smith, 1920), often with characteristic whorls in the centre. In contrast, non-mucoid colonies are opaque, tarnished, and show a round shape. All *R. solanacearum* colonies are non-fluorescent, although a diffusible brown pigment can be produced on some media (Denny & Hayward, 2001). Moreover, on the nutrient general media currently recommended by EU Directives *R. solanacearum* fluidal colonies are pearly cream-white and the afluidal ones are entirely cream-white, whereas on semi-selective media fluidal colonies have whorls blood red in colour and afluidal ones can be entirely deep red (Anonymous, 1998, 2006) (Fig. 1.4).

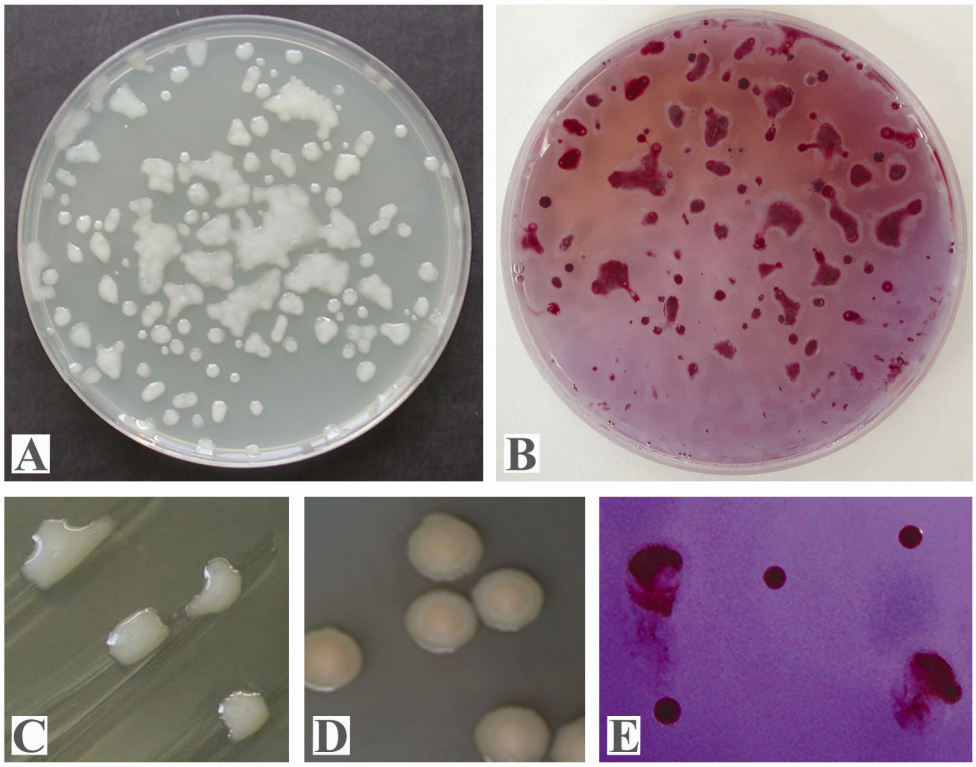


Figure 1.4. *R. solanacearum* colony morphology on (A) YPGA, a non-selective Yeast Peptone Glucose Agar medium, and (B) SMSA, a modified Semiselective Medium South Africa agar. Detail of fluidal colonies (C) and afluidal ones (D) on YPGA, and fluidal and afluidal colonies on SMSA (E). Both media are recommended by EU Directives. [(A, B and E) B. Álvarez, (C and D) P. Caruso].

Under certain conditions, *R. solanacearum* colonies spontaneously undergo a change from fluidal to afluidal morphology, linked to a great reduction in disease-inducing capacity of these cells (Kelman, 1954; Buddenhagen & Kelman, 1964; Brumbley & Denny, 1990). This phenomenon is known as "Phenotypic Conversion" (PC) (Denny *et al.*, 1994; Poussier *et al.*, 2003) and occurs in most if not all strains of *R. solanacearum* (Brumbley & Denny, 1990). *R. solanacearum* PC-type variants can be easily observed by prolonged culture on agar plates (Kelman, 1954; Buddenhagen & Kelman, 1964), and also when the organism is grown in a still, non-aerated liquid medium with glucose and an organic source of nitrogen (Kelman & Hruschka, 1973). Morphological characteristics of PC-type colonies are those already described for the non-mucoid colonies of *R. solanacearum*, namely afluidal and round.

Flagellation of *R. solanacearum* has reportedly been a confusing characteristic, which caused the bacterium to be placed in different genera (see section 1.2.1). Although early reports described it as motile, polar flagellated (Smith, 1920; Kelman, 1953; Hodgkiss, 1964), this was later on suspected to be due to the rapid appearance of spontaneous motile avirulent mutants in culture (Kelman & Hruschka, 1973), and concluded that the bacterium was non-motile, non-flagellated (Brumbley & Denny, 1990; Yabuuchi *et al.*, 1995). More recently, Clough *et al.* (1997) reconciled these contradictions by demonstrating that motility is transiently expressed during *R. solanacearum* growth. Thus, the bacterium can produce one to several polar flagella (Smith, 1920; Kelman, 1953; Hodgkiss, 1964; Clough *et al.*, 1997), which provide it with swimming motility (Tans-Kersten *et al.*, 2001). However, to observe this motility *R. solanacearum* cell density should be $>10^6$ and $<10^9$ cfu/ml (Clough *et al.*, 1997). This would be related to the culture age, so that high numbers of motile cells could be obtained in exponential phase, whereas in stationary phase there would be a majority of non-motile (Clough *et al.*, 1997). In accordance with that, motility was needed to effectively invade and colonize the host, although cells were non-motile in host xylem vessels (Tans-Kersten *et al.*, 2001). It was also found that cells from wilted plants were non-motile, but became motile after a few hours in fresh medium (Mao & He, 1998).

R. solanacearum exhibits twitching motility over solid surfaces (Henrichsen, 1983; Liu *et al.*, 2001; Kang *et al.*, 2002), with race 1 and 2 strains generally twitching more actively than those of race 3 (Liu *et al.*, 2001). This form of motility consists of a flagella-independent translocation requiring retractile type IV pili (fimbriae). Flexuous polar pili of this type have been demonstrated to be present on *R. solanacearum* (Kang *et al.*, 2002) and be related to twitching motility (Liu *et al.*, 2001; Kang *et al.*, 2002), adherence to various surfaces and natural transformation (Kang *et al.*, 2002). Moreover, *R. solanacearum* variants having mutations in type IV pili were markedly less virulent on host plants, and consequently motility, adherence and/or type IV pili appear to have a significant role in *R. solanacearum* pathogenesis (Liu *et al.*, 2001; Kang *et al.*, 2002).

1.2.3 Diversity

R. solanacearum is currently considered a heterogeneous species or a "species-complex" (Fegan & Prior, 2005), supporting the concept of "species-group" already proposed by Buddenhagen and Kelman in 1964, perhaps on the hypothesis that strains of this species "are the product of long evolution occurring independently in various areas on different hosts" (Buddenhagen & Kelman, 1964).

Apart from races (see section 1.1.2), *R. solanacearum* strains have been traditionally classified into biovars, on the basis of the pathogen ability to utilize and/or oxidize several hexose alcohols and disaccharides (Hayward, 1964, 1991). So far, five races (Table 1.2) and biovars (Tables 1.3 and 1.4) have been described for the bacterium (Buddenhagen & Kelman, 1964; Hayward, 1991; EPPO, 2004). Both classification schemes constitute informal groupings at the infrasubspecific level (Hayward, 1991). The relationship between them is only evident with race 3 strains, which generally correlate with biovar 2 phenotype (Buddenhagen & Kelman, 1964; Hayward, 1991).

Table 1.2. Races of the *R. solanacearum* species complex. [From Denny & Hayward, 2001].

RACE	HOST RANGE	GEOGRAPHICAL DISTRIBUTION
1	Wide	Asia, Australia, Americas
2	Banana, other *Musa* spp	Caribbean, Brazil, Philippines
3	Potato	Worldwide
4	Ginger	Asia
5	Mulberry	China

Table 1.3. Biovars of the *R. solanacearum* species complex. [From Denny & Hayward, 2001].

TEST	BIOVAR				
	1	2	3	4	5
Dextrose	+	+	+	+	+
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-
Dulcitol	-	-	+	+	-
Trehalose	+	-	+	+	+
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
D(+) cellobiose	-	+	+	-	+
Nitrite from nitrate	+	+	+	+	+
Gas from nitrate	-	-	+	+	+

Table 1.4. Biovar 2 of the *R. solanacearum* species complex. [From Anonymous, 2006].

TEST	BIOVAR		
	2A (Worldwide)	**2A (Chile Colombia)**	**2T (Tropical areas)**
Utilisation of trehalose	-	+	+
Utilisation of meso-inositol	+	-	+
Utilisation of D-ribose	-	-	+
Pectolytic activity	low	low	high

Several molecular techniques have been applied to study diversity within *R. solanacearum* since the last twenty years. Restriction fragment length polymorphism (RFLP) analysis and other genetic fingerprinting tests (Hayward, 2000) showed groups included in two divisions for this species: division I, comprising strains belonging to biovars 3, 4 and 5, primarily isolated in Asia, and division II, with strains of biovars 1, 2A and 2T, isolated from America (Cook *et al.*, 1989; Cook & Sequeira 1994). Race 3 appears to be mostly biovar 2A RFLP group 26 with a worldwide distribution (Cook & Sequeira, 1994), biovar 2A RFLP group 27 (found in Chile and Colombia), or biovar 2T (sometimes also called 2N, found in tropical areas in South America) (EPPO, 2004). Polymerase chain reaction (PCR) amplification and restriction analysis confirmed these two divisions (Seal *et al.*, 1992; Gillings *et al.*, 1993).

More recently, a classification of the *R. solanacearum* species into phylotypes has been established (Fegan & Prior, 2005). A phylotype corresponds to a monophyletic cluster of strains as determined after phylogenetic analysis of sequences of the 16S-23S rRNA gene intergenic spacer (ITS) region, the *hrpB* gene and the endoglucanase (*egl*) gene (Fegan & Prior, 2005). Thus, the species can be divided into four phylotypes corresponding to four broad genetic groups, each of them related to a geographic origin (Fegan & Prior, 2005) (Fig. 1.5). Phylotype I contains all

strains belonging to biovars 3, 4 and 5, isolated primarily from Asia. Phylotype II includes biovar 1, 2 and 2T strains isolated from America, all race 3 strains pathogenic to potato and the race 2 banana pathogen. Phylotype III comprises strains belonging to biovars 1 and 2T, from Africa and surrounding islands. Phylotype IV is more heterogeneous, with biovar 1, 2 and 2T strains from Indonesia, strains isolated in Australia and Japan, and also *R. syzygii* and the blood disease bacterium (BDB) (Fegan & Prior, 2005).

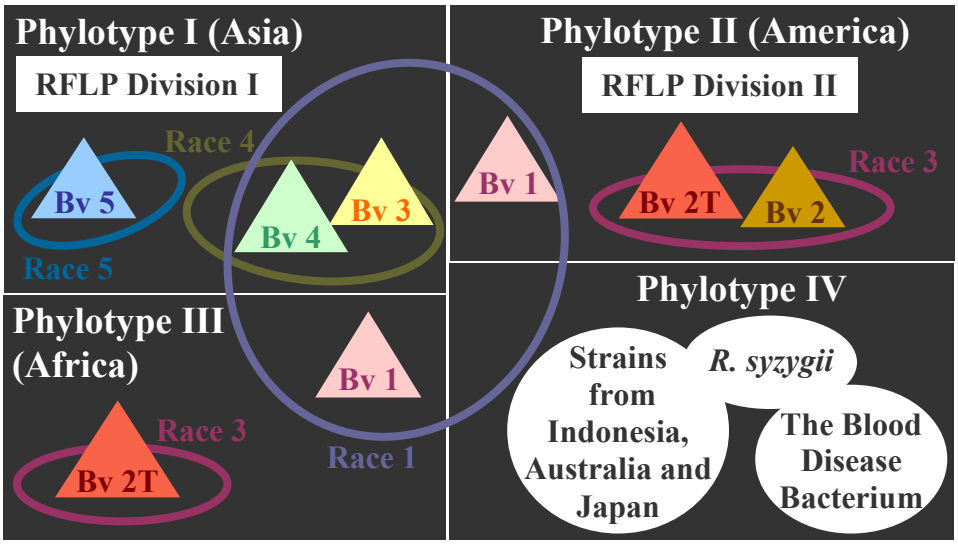


Figure 1.5. Diverse classification systems of the *R. solanacearum* species complex and their correspondence. [From Fegan & Prior, 2005; adapted by Álvarez, 2009].

A subsequent study on genetic diversity of a collection of *R. solanacearum* strains confirmed the four major, deeply separated evolutionary phylotypes, with a weaker subdivision of phylotype II into two subgroups, and reported geographic isolation and spatial distance as the significant determinants of genetic variation between the phylotypes (Castillo & Greenberg, 2007). According to the study, genes essential for survival would be under purifying or stabilizing selection (that reduces the frequency of deleterious alleles until they are eliminated from the population), while those involved in pathogenesis may be under diversifying selection (that generates multiple alleles that can remain for

long periods in the population, and induces polymorphism) (Castillo & Greenberg, 2007).

The diverse classification schemes proposed for *R. solanacearum* (Fig. 1.5) reflect the great phenotypic and genotypic variation within the species, which has led to coin the term "*R. solanacearum* species complex" (Gillings & Fahy, 1993), defined as "a cluster of closely related isolates whose individual members may represent more than one species" (Fegan & Prior, 2005).

1.2.4 Genome

In the last years, the genomes of three *R. solanacearum* strains with very different host ranges have been sequenced at Genoscope (http://www.genoscope.cns.fr), in France. The first was that of strain GMI 1000 (Salanoubat *et al.*, 2002), isolated from a tomato plant with bacterial wilt symptoms in Guyana (Boucher *et al.*, 1985), and classified as phylotype I, race 1, biovar 3. It has a wide host range and was selected because it is able to infect the model plant *Arabidopsis thaliana*, whose genome has also been entirely sequenced, both of them therefore allowing the study of host-pathogen interaction at the genomic scale.

The genome of the *R. solanacearum* strain GMI 1000 has a size of 5.8 Mb with a high G+C content (average value of 67%) and a coding potential for approximately 5120 proteins. It is organized in two independently replicating circular replicons: a 3.7 Mb chromosome and a 2.1 Mb megaplasmid (Genin & Boucher, 2002, 2004) (Fig. 1.6). The chromosome harbors genes for basic cellular functions. The megaplasmid encodes many essential pathogenicity genes, as well as genes that may be related to the overall fitness of the bacterium, like those of copper and cobalt-zinc-cadmium resistance, catabolism of aromatic compounds and flagelum biosynthesis (Genin & Boucher, 2002). The megaplasmid carries duplications of some important genes (for instance, a rDNA locus, three tRNAs, a second sigma54 factor gene, and a second subunit alfa of DNA polymerase III) but, also genes with no counterpart on the cromosome (for instance, genes encoding enzymes involved in the metabolism of small molecules i.e. methionine biosynthesis). On the basis of these data, Genin

and Boucher (2002) speculated that the megaplasmid would be in the process of acquiring new functions through duplication or translocation of essential genes from the chromosome.

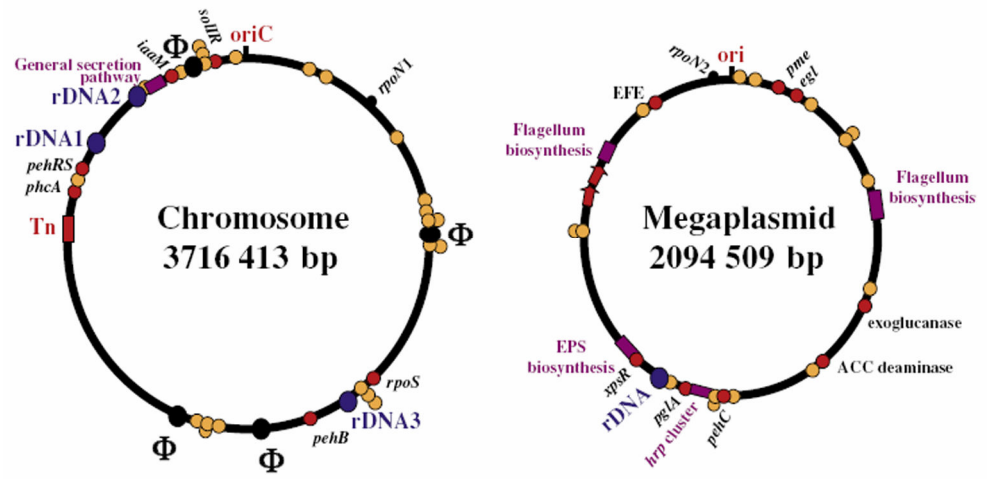


Figure 1.6. Organization of the *R. solanacearum* genome, strain GMI 1000. [From Genin & Boucher, 2002].

According to Genoscope, the genomes of two other strains of *R. solanacearum*, Molk 2 and IPO 1609, are being sequenced on regions of special interest, in each of them. Although both strains belong to phylotype II, Molk 2 is a strain of race 2 isolated from a banana tree and strictly limited to this host, whereas IPO 1609 is a race 3 strain isolated from potato in The Netherlands (van Elsas *et al.*, 2001) and considered to be the representative strain for *R. solanacearum* strains introduced to Europe, mainly affecting solanaceous plants. The genomes of Molk 2 and IPO 1609 are comparable to that of GMI 1000 (about 6 Mb, GC% estimated at 67%) although, when approximately 100 kb from the Molk 2 genome were sequenced at random and compared with GMI 1000 genome, up to 30% of the genome of Molk 2 was absent in the genome of GMI 1000 (Genoscope, 2008).

In the United States a draft genome of *R. solanacearum* strain UW551, a race 3 biovar 2 strain isolated from geranium, was obtained and annotated (Gabriel *et al.*, 2006). GC content was approximately 64.5%, and about 62% of the open reading frames (ORFs) had a functional assignment

(Gabriel *et al.*, 2006). The sequences were compared with the genome of strain GMI 1000 (Salanoubat *et al.*, 2002). Most genes encoding pathogenicity determinants appeared to be common to both strains, while one 22-kb region present in GMI 1000 apparently by horizontal gene transfer was absent from UW551 and likely encodes enzymes essential for utilization of the three sugar alcohols that distinguish biovars 3 and 4 from biovars 1 and 2 (Gabriel *et al.*, 2006).

In spite of the existence of a core genome in the four strains, some genomic instability has been reported for *R. solanacearum* (Kelman, 1954; Boucher *et al.*, 1988). In fact, *R. solanacearum* genome harbors many transposable elements that may take active part in acquisition, loss and alteration of genetic material, and so contributing to generate genomic variation (Genin & Boucher, 2002, 2004). This flexibility would be related to the great diversity within the *R. solanacearum* species complex.

Sequencing of these genomes has permitted the identification of a number of candidate genes for pathogenesis including those related to biosynthesis of lytic enzymes and EPS, hypersensitive reaction and pathogenicity (*hrp*) genes, structural genes encoding "effector" proteins injected by the bacterium into the plant cell by a type III secretion system (T3SS), and enzymes controlling plant hormone production, factors implicated in cell adherence, and others. Nowadays, research is aimed to the functional analysis of effectors and the characterization of their molecular targets in the plant cell.

1.2.5 Pathogenicity determinants

R. solanacearum secretes many extracellular products and possesses diverse genes involved in colonization and wilting of host plants (Denny *et al.*, 1990; Schell, 2000; Genin & Boucher, 2002, 2004). The most important of them have been summarized below.

1.2.5.1 Hydrolytic enzymes

Phytopathogenic bacteria have often developed enzymes to hydrolize plant cell wall components, since they can represent an important source of nutrients and energy. These enzymes are further

involved in early stages of the infective process, favouring the entry and advance of the pathogenic agent in host tissues (Boucher *et al.*, 2001). *R. solanacearum* produces several plant cell wall-degrading enzymes, secreted via the type two secretion system (T2SS) (Huang & Allen, 1997; Tans-Kersten *et al.*, 1998). These include one β-1,4-cellobiohydrolase (CbhA) and some pectinases whose activities have been identified respectively as one β-1,4-endoglucanase (Egl) (Schell, 1987; Roberts *et al.*, 1988), one endopolygalacturonase (PehA), two exopolygalacturonases (PehB and PehC) (Schell *et al.*, 1988; Huang & Allen, 1997), and one pectin methyl esterase (Pme) (Tans-Kersten *et al.*, 1998). *R. solanacearum* Egl is a 43-kd protein (Schell, 1987) that has proved to be involved in pathogenicity (Roberts *et al.*, 1988) as previously suggested (Kelman & Cowling, 1965). Inactivation of *egl*, *pehA* or *pehB* genes revealed that each contribute to *R. solanacearum* virulence, and a deficient mutant lacking the six enzymes wilted host plants more slowly than the wild-type (Liu *et al.*, 2005). Since pectin catabolism does not significantly contribute to bacterial fitness inside the plant (González & Allen, 2003), it seems that cellulase and pectinolytic activities are preferably required for host colonization than for bacterial nutrition (Valls *et al.*, 2006). Thus, *R. solanacearum* hydrolytic enzymes are thought to be involved in virulence *in planta* (Hikichi *et al.*, 2007).

1.2.5.2 Exopolysaccharide

Several phytopathogenic bacterial species produce high amounts of EPSs either in pure culture or during *in planta* multiplication. Although usually related to pathogenicity, it is often difficult to know if the EPSs take active part in symptom production or if they indirectly favour infection. In *R. solanacearum*, it has been reported that all virulent wild-type strains (mucoid colonies) produce EPS (Kelman, 1954; Husain & Kelman, 1958; Buddenhagen & Kelman, 1964; Boucher *et al.*, 1992; Poussier *et al.*, 2003), while EPS-deficient mutants (non-mucoid colonies) are avirulent.

R. solanacearum EPS appears to be highly heterogeneous, since it has a varying composition among strains (Drigués *et al.*, 1985). The main virulence factor is an acidic, high molecular mass extracellular polysaccharide (EPS I), a long (>10^6 Da) polymer with a trimeric repeat unit

of N-acetyl galactosamine, 2-N-acetyl-2-deoxy-L-galacturonic acid, and 2-N-acetyl-4-N-(3-hydroxybutanoyl)-2-4-6-trideoxy-D-glucose (Orgambide *et al*., 1991; Schell *et al*., 1993; Schell, 2000). EPS I is more than 90% of the total *R. solanacearum* EPS produced (Schell *et al*., 1993; McGarvey *et al*., 1998, 1999), and approximately 85% appears as a released, cell-free slime, whereas 15% has a cell surface-bound capsular form (McGarvey *et al*., 1998; Schell, 2000). In studies carried out with EPS I-deficient mutants, it was found that EPS I caused wilting in infected plants (Denny & Baek, 1991; Kao *et al*., 1992; Denny, 1995; Saile *et al*., 1997; McGarvey *et al*., 1999).

In planta, EPS would probably act by occluding xylem vessels, interfering directly with normal fluid movement of the plant (Husain & Kelman, 1958; Denny, 1995) or by breaking the vessels due to hydrostatic overpressure (van Alfen, 1989). On the other hand, EPS I might also favour stem colonization by the pathogen, since EPS I-deficient mutants were shown to multiply more slowly, and colonized poorly the stem of infected plants (Saile *et al*., 1997; Araud-Razou *et al*., 1998). In that sense, EPS I would be contributing to minimizing or avoiding the recognition of bacterial surface structures such as pili and/or lipopolysaccharide by plant defence mechanisms (Duvick & Sequeira, 1984; Young & Sequeira, 1986; Denny, 1995; Araud-Razou *et al*., 1998). As EPS-deficient mutants can infect and multiply to some extent *in planta* without inducing wilting symptoms, EPS might take part mainly in late stages of the process, modulating disease severity rather than the infective ability of the strain. In *R. solanacearum*, EPS is thought to be the main factor accounting for the virulence of the pathogen.

1.2.5.3 *Hrp* genes

Screening for pathogenicity-deficient mutants after random mutagenesis of complete genomes led to the discovery of *hrp* mutants, unable to induce symptoms in susceptible host plants and a hypersensitive reaction (HR) in resistant plants or non-hosts (Boucher *et al*., 1992). H*rp* gene clusters have been identified in several plant pathogenic bacteria, where two lineages have been described: group I for *Erwinia* sp., *Pantoea*

stewartii, and *Pseudomonas syringae*, and group II for *R. solanacearum* and *Xanthomonas* sp. (Alfano & Collmer, 1997; He *et al.*, 2004; Meyer *et al.*, 2006).

In *R. solanacearum*, *hrp* genes control both disease development and HR induction (Boucher *et al.*, 1987, 1992, 2001). HR is a plant defense mechanism preventing the spread of pathogen infection to other parts of the plant. It is associated with plant resistance and characterized by a rapid and programmed plant cell death localized in the region surrounding an infection (Nimchuk *et al.*, 2003). It can be macroscopically visualized as necrotic areas in the plant tissues affected, generally in tobacco plants.

Hrp mutants do not seem to be involved in the infection process, since most of them could be isolated from the stems of infected plants (Trigalet & Démery, 1986). The mutants showed an impaired ability to multiply *in planta* (Trigalet & Démery, 1986), not observed when cultured on minimum media (Boucher *et al.*, 1985), which may indicate a possible role of *hrp* genes in diverting certain plant metabolites from the plant to the bacteria (Boucher *et al.*, 1992).

Detailed genetic analysis of *hrp* mutants revealed more than 20 *hrp* genes clustered on a region of 20-25 kb of the bacterial megaplasmid (Genin & Boucher, 2004) (Fig. 1.7). The cluster encodes components of a T3SS (van Gijsegem *et al.*, 1993) and effector proteins. In all *hrp* clusters, eleven conserved genes (*hrc* genes) might be forming the core of the T3SS (Bogdanove *et al.*, 1996).

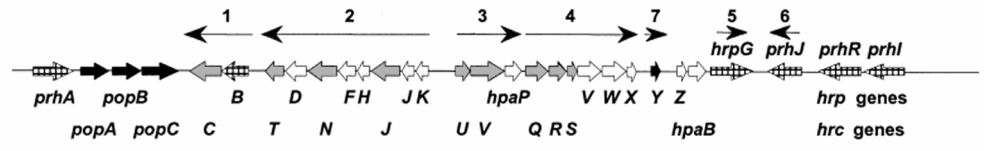


Figure 1.7. Genetic organization of the *R. solanacearum hrp* gene cluster. The thin arrows with numbers indicate the *hrp* transcription units; the thick arrows indicate the different genes. Conserved *hrc* genes are represented by filled grey arrows, genes encoding secreted proteins by filled black arrows, and regulatory genes by hatched white arrows. [From van Gijsegem *et al.*, 2002].

T3SSs have an important role in pathogenesis, since they are capable of delivering proteins into the cytosol of eukaryotic cells (van Gijsegem *et al.*, 1995; Cornelis & van Gijsegem, 2000; Genin & Boucher, 2002; He *et al.*, 2004) (Fig. 1.8). These systems are thought to secrete effector proteins translocated inside host cells and accessory proteins supporting the translocation or translocators (Petnicki-Ocwieja *et al.*, 2005; Meyer *et al.*, 2006). Effector proteins would act in the invasive stages of the infection in order to favour bacterial development by either inhibiting plant defenses or inducing nutrient release from the host cell (Boucher *et al.*, 1992, 2001; Meyer *et al.*, 2006). Moreover, in some cases effectors can elicit HR due to recognition by specific plant resistance genes. These effectors are then referred to as avirulence (Avr) proteins, since they would be hindering pathogenicity on the host having a corresponding resistance gene (Keen, 1990).

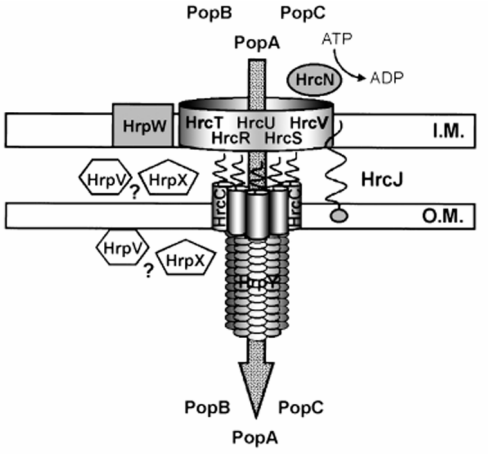


Figure 1.8. *R. solanacearum* type III secretion apparatus. [From van Gijsegem *et al.*, 2002].

T3SS structure includes extracellular appendages as the Hrp pili in plant pathogens, believed to function either in the attachment to plant cells and/or as conduits for protein translocation, since they might penetrate the plant cell wall. *In vitro*, Hrp pili-deficient mutants were impaired in secretion of effectors and accessory proteins (He *et al.*, 2004). *R. solanacearum* produces Hrp-dependent pili, in addition to the polar fimbriae which were independent on the expression of the *hrp* genes (see section

1.2.2), although both types of pili are located at the same pole of the bacterium (van Gijsegem *et al.*, 2000) (Fig. 1.9). *R. solanacearum* Hrp pili are mainly composed of the HrpY protein, essential for T3 protein secretion but, dispensable for attachment to plant cells (van Gijsegem *et al.*, 2000). More recently, two proteins secreted via the T3SS, PopF1 and PopF2, were identified as translocators, with PopF1 playing a more important role in virulence and HR elicitation than PopF2 (Meyer *et al.*, 2006) (Fig. 1.10).

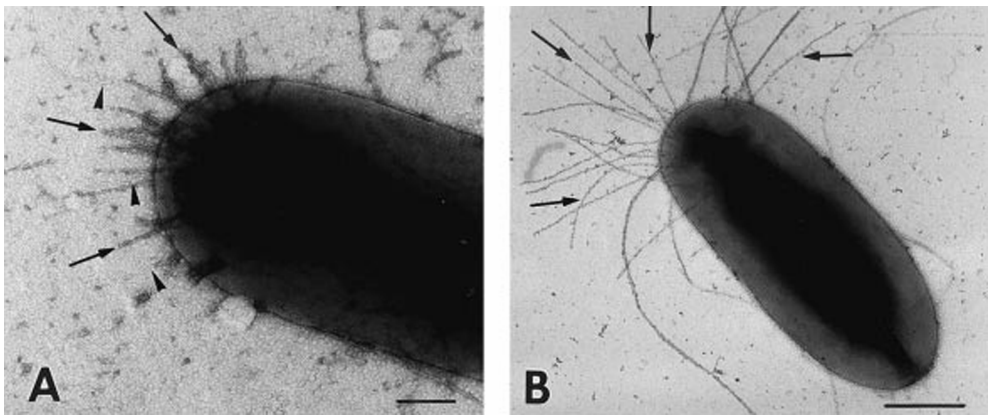


Figure 1.9. Hrp-pilus of *R. solanacearum* strain GMI 1000 at different times of growth. (A) Numerous short and straight Hrp-pili (arrows) and few fimbriae (arrowheads) are present at the same pole of the bacterium. Bar represents 0.2 mm. (B) Elongated Hrp-pili (arrows) are observed. Bar represents 0.5 mm. [From van Gijsegem *et al.*, 2000].

R. solanacearum T3SS secretes proteins PopA, PopB, PopC and PopP1 into the extracellular medium under control of the transcriptional regulator HrpB. PopA produces a hypersensitive-like response when infiltrated into plant tissue at high concentration (Arlat *et al.*, 1994). It has been suggested that this protein may allow nutrient acquisition *in planta* and/or the delivery of effector proteins into plant cells (Lee *et al.*, 2001). PopB has a nuclear localization signal which enables this protein to be transported to the plant cell nucleus (Guéneron *et al.*, 2000). PopC contains sequences analogous to those of some plant resistance gene products (Guéneron *et al.*, 2000). PopP1 acts as an *avr* determinant towards resistant plants (Lavie *et al.*, 2002). PopA, PopB, PopC or PopP1-deficient mutants

showed normal virulence in several different host plant species tested, probably due to functional redundancy (Guéneron *et al.*, 2000; Cunnac *et al.*, 2004). Five candidate effector proteins were shown to be translocated into host cells by the *R. solanacearum* T3SS, and 48 novel *hrpB*–regulated genes have been identified, with half of them encoding novel classes of probable effector proteins with no counterparts in other bacterial species (Cunnac *et al.*, 2004).

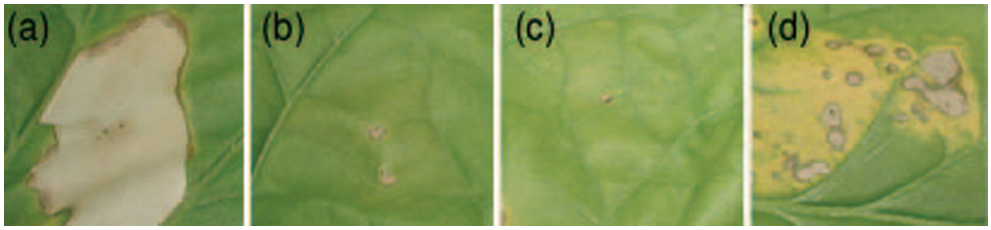


Figure 1.10. HR tests in tobacco leaves of *R. solanacearum* (a) strain GMI 1000, (b) *hrpB* mutant, (c) *popF1 popF2* double mutant, and (d) complementated mutant. [From Meyer *et al.*, 2006].

1.2.5.4 Other pathogenicity determinants

Lipopolysaccharide (LPS). The recognition between *R. solanacearum* and the host has long been thought to implicate an interaction between bacterial LPS, a component of the outer membrane, and plant lectins. Therefore, *R. solanacearum* LPS is significantly involved in the virulence of this organism (Whatley *et al.*, 1980; Baker *et al.*, 1984; Hendrick & Sequeira, 1984). It has three parts: the lipid A, the oligosaccharide core and the O-specific antigen (Baker *et al.*, 1984). The core structure is composed of rhamnose, glucose, heptose, and 2-ketodeoxy-octonate, whereas the O-specific antigen is a chain of repeating rhamnose, N-acetylglucosamine, and xylose in a ratio of 4:1:1 (Baker *et al.*, 1984). Presence or absence of the O-specific antigen differentiated respectively between smooth and rough LPSs, appearing in *R. solanacearum* strains which were respectively negative and positive HR-inducers (Whatley *et al.*, 1980; Baker *et al.*, 1984). However, subsequent research indicated that a specific interaction between *R. solanacearum* rough LPS and a plant cell wall receptor was not enough to

iniciate the HR, although many of the mutations in the LPS also affected virulence (Hendrick & Sequeira, 1984). In *R. solanacearum*, smooth LPS apparently is required to prevent agglutination by certain plant lectins (Sequeira & Graham, 1977). Furthermore, *R. solanacearum* LPS and EPS are somehow related, since a gene cluster was found to be required for the biosynthesis of both cell surface components (Kao & Sequeira, 1991).

Lectins. Two genes encoding lectins have been characterised in *R. solanacearum* (Sudakevitz *et al.*, 2004; Kostlanova *et al.*, 2005), presumably with a function in adhesion to plant surfaces, which is important for *R. solanacearum* pathogenicity during the early stages of infection. In fact, it was found that these lectins bind L-fucose and interact with the plant xyloglucan polysaccharide belonging to the hemicellulose fraction of plant primary cell walls (Kostlanova *et al.*, 2005).

Siderophores. Although it seems to be strongly dependent on the strain (Bhatt & Denny, 2004), *R. solanacearum* produces the dihydroxamate citrate-containing siderophore schizokinen (Budzikiewicz *et al.*, 1997) and the polycarboxylate siderophore staphyloferrin B (Bhatt & Denny, 2004). However, siderophores appear to be non-essential for virulence of the pathogen *in planta*, probably because iron concentration in the xylem is high enough to supress the production, and the iron is in a molecular form that the bacterium can utilize (Bhatt & Denny, 2004).

1.2.6 Control of main pathogenicity genes

The pathogenicity of *R. solanacearum* is differently regulated in the early and late stages of infection in response to environmental conditions, such as presence of host plant cells and bacterial population densities (Schell, 2000; Hikichi *et al.*, 2007) (Fig. 1.11).

1.2.6.1. Control in early stages of infection

When *R. solanacearum* is in the presence of a plant cell, plant-pathogen physical interaction is sensed in the bacterium by an outer membrane receptor, PrhA, which transduces the signals through a complex regulatory cascade progressively integrated by PrhR, PrhI, PrhJ, HrpG and

HrpB regulators (Brito *et al.*, 1999, 2002; Aldon *et al.*, 2000; Cunnac *et al.*, 2004; Hikichi *et al.*, 2007) (Fig. 1.11). The nature of the cell wall inducer is not known although it is likely to involve a carbohydrate from the pectic/cellulosic portion (Aldon *et al.*, 2000). Among the components of the transducing cascade, HrpB is a major regulator for *R. solanacearum* pathogenicity since it activates expression of the *hrp* genes which encode the T3SS (Schell, 2000; Genin & Boucher, 2002, 2004).

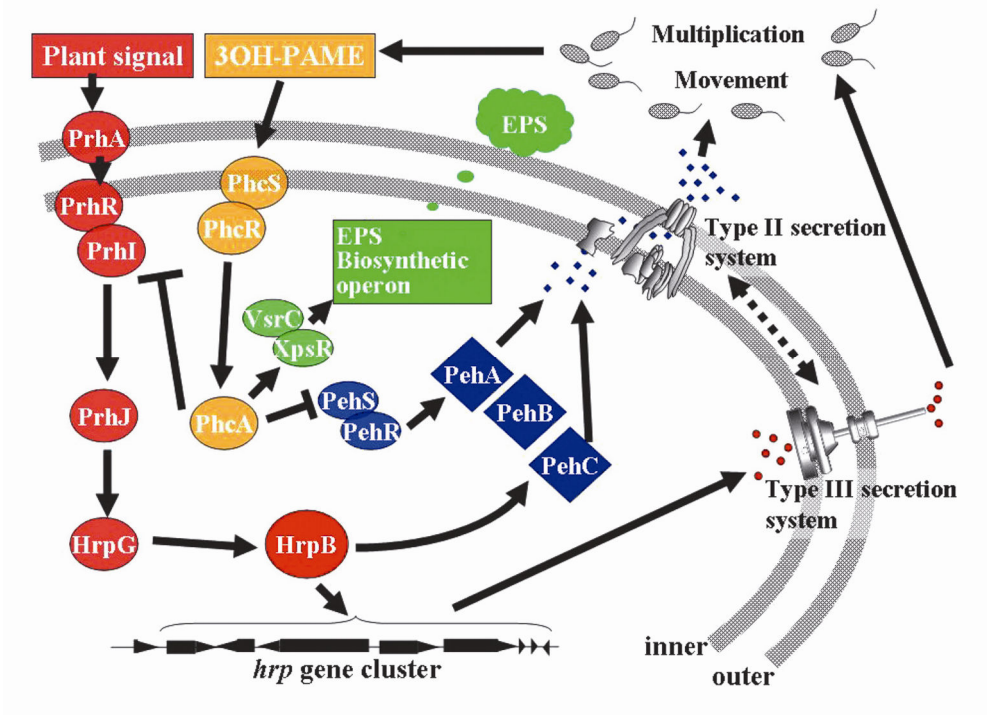


Figure 1.11. Regulation of pathogenicity-related genes in *R. solanacearum* in early stages of infection (red pathway), and in late stages of infection (orange pathway). [From Hikichi *et al.*, 2007].

R. solanacearum is the bacterial phytopathogen for which *hrp* gene regulation is best known (Boucher *et al.*, 2001). Expression is environmentally responsive to at least two external signals. The first one is a minimal medium mimicking that of the intercellular spaces *in planta*. It has been shown that expression of *R. solanacearum hrp* cluster is repressed during growth in rich medium and activated in minimal medium, and therefore it depends on the nutritional status of the organism (Boucher *et*

al., 1992). Moreover, the nature of the carbon source in minimal medium has an influence on the level of *hrp* gene expression. In that sense, glucose amended with some plant extracts mildly stimulated transcription, whereas pyruvate yielded levels comparable to those observed *in planta* (Arlat *et al.*, 1992). The second one is a non-diffusible specific inducing signal perceived in the presence of plant cell walls (Marenda *et al.*, 1998; Schell, 2000; Brito *et al.*, 2002).

Thus, when *R. solanacearum* is in physical contact with plant cells or cell wall fragments, expression of the regulatory gene *hrpB* is maximally induced (Aldon *et al.*, 2000; Boucher *et al.*, 2001). Translocators PopF1 and PopF2 are regulated by HrpB (Meyer *et al.*, 2006) and so effector proteins to be injected out of the bacterial cell (Genin & Boucher, 2002, 2004; Cunnac *et al.*, 2004; Hikichi *et al.*, 2007). In consequence, transcriptional activation of *hrp* genes by HrpB allows the pathogen to invade the host and proliferate in intercellular spaces (Hikichi *et al.*, 2007). During this stage of the infection, T2SS seems to cooperate with T3SS in terms of co-regulation of pathogenicity factors and, for instance, T2SS would influence secretion of PopB, while the *pehC* gene is positively regulated by HrpB (Hikichi *et al.*, 2007). Furthermore, transcriptome analysis of a *hrpB*-deficient mutant revealed that HrpB action extends beyond the T3SS (Occhialini *et al.*, 2005). In that sense, it has recently been reported that this regulator activates an operon of six genes responsible for the synthesis of a HrpB-dependent factor (HDF), a tryptophan derivative presumably involved in acyl-homoserine lactone receptor-mediated activity (Delaspre *et al.*, 2007) (Fig. 1.12).

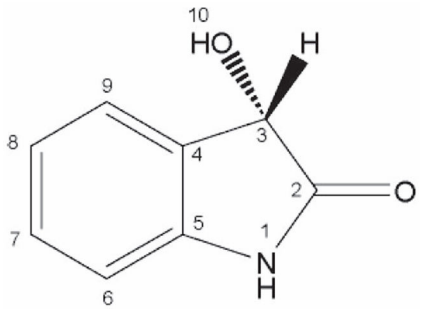


Figure 1.12. Chemical structure of HDF. [From Delaspre *et al.*, 2007].

Acyl-homoserine lactones are autoinducers taking part in quorum sensing systems of bacteria. This kind of systems has been described as regulatory networks employing autoregulators to activate gene expression only at high cell densities, and therefore they can mediate long-distance intercellular communication (Fuqua *et al.*, 1996; Flavier *et al.*, 1997).

1.2.6.2. Control in late stages of infection

During colonization *in planta*, T2SS also contributes to *R. solanacearum* invasion into xylem vessels, resulting in systemic infection of the host (Hikichi *et al.*, 2007) (Fig. 1.11). At this stage, production of extracellular pathogenicity determinants is transcriptionally controlled by an extensive and complex regulatory network of distinct, interacting signal transduction pathways (Schell, 2000). The main transcriptional regulator is PhcA (Phc, phenotype conversion) (Brumbley *et al.*, 1993), which simultaneously activates diverse virulence genes such as those of EPS biosynthesis and production of Pme and Egl exoproteins, and represses others such as *hrp* genes and those related to production of polygalacturonases and siderophores, and motility (Schell, 2000; Genin & Boucher, 2002). PhcA is involved in the five-gene cell density-sensing system (Schell, 2000), an essential part of the complex regulatory network, which controls activity of PhcA protein in response to levels of an endogenous signal molecule, the 3-hydroxypalmitic acid methyl ester (3-OH PAME) (Schell, 2000). Thus, at low bacterial cell densities, for instance, outside the host or in the early stages of host colonization, 3-OH PAME is not produced, PhcA regulator remains inactive and *R. solanacearum* cells are motile and highly pectolytic. When bacterial cell densities are high, as in the xylem vessels of the host, 3-OH PAME accumulates extracellularly, promoting the activation of PhcA production, which results in activation of EPS and hydrolytic enzyme synthesis and repression of motility, when 3-OH PAME has exceeded a threshold (Flavier *et al.*, 1997a; Schell, 2000; Genin & Boucher, 2002). 3-OH PAME is then an intercellular signal autoregulating virulence gene expression in *R. solanacearum*, acting in the quorum-sensing system of this pathogen (Flavier *et al.*, 1997). The PhcA regulator also positively controls production of a second quorum-sensing

molecule in *R. solanacearum*, an acylhomoserine lactone (Flavier *et al.*, 1997b), which takes part in an additional regulatory system mediated by the SolI-SolR regulators to fine-tune virulence gene expression (Flavier *et al.*, 1997b; Schell, 2000). Moreover, *R. solanacearum* megaplasmid has a pair of ORFs showing homology to SolI-SolR, what suggest the existence of a third quorum-sensing system in the pathogen (Genin & Boucher, 2002).

Mutation in *phcA* results in the phenotypic conversion of *R. solanacearum* colonies into the afluidal type (Brumbley & Denny, 1990; Brumbley *et al.*, 1993; Denny *et al.*, 1994; Jeong & Timmis, 2000). The conversion involves the loss of one or more components of the EPS (Husain & Kelman, 1958; Orgambide *et al.*, 1991) and a strong reduction in endoglucanase activity but an increase in that of endopolygalacturonase (Schell, 1987; Brumbley & Denny, 1990). Furthermore, cell motility has been shown to be increased in PC-type variants (Kelman & Hruschka, 1973; Brumbley & Denny, 1990). Although these variants are impaired in wilting host plants (Kelman, 1954), they are infective and can grow *in planta* (Denny & Baek, 1991), causing disease symptoms such as stunting, stem necrosis and adventitious root formation (Husain & Kelman, 1958; Denny & Baek, 1991). Several PC-type variants carrying localized mutations within *phcA* were able to revert from PC to wild type *in planta*, and also *in vitro* when in presence of root exudates of susceptible hosts (Poussier *et al.*, 2003).

1.2.6.3. Control by HrpG

As mentioned, HrpG is a host-responsive regulator of the *R. solanacearum* T3SS activation cascade. It has recently been demonstrated that HrpG actually co-regulates the expression of two independent pathways (Valls *et al.*, 2006) (Fig. 1.13). The first one is that dependent on HrpB, comprising the T3SS cascade (Cunnac *et al.*, 2004; Occhialini *et al.*, 2005). The second one is independent of HrpB and controls the expression of genes likely involved in adaptation to life *in planta* (Valls *et al.*, 2006). Since a hrpG-deficient mutant constitutively expressing the *hrpB* gene was strongly altered in its ability to wilt host plants, this second pathway is also essential for full *R. solanacearum* virulence (Valls *et al.*, 2006), as it had been

hypothesized (Vasse *et al.*, 2000). HrpG has thus been proposed to have a role in the molecular transition between the environment and the host by shifting the expression of a large set of genes in addition to those concerning the T3SS (Valls *et al.*, 2006). According to these authors, the second pathway co-ordinates bacterial production of plant cell wall degrading enzymes, EPS and two phytohormones, ethylene and auxin, known to be produced by the pathogen (Freebairn & Buddenhagen, 1964; Sequeira & Williams 1964). Thus, the ethylene produced by *R. solanacearum* is sufficient to modulate the expression of host genes, interfering with the signalling of plant defence responses to favour infection during the early stages (Valls *et al.*, 2006). Moreover, a co-ordinated production of T3SS effectors and ethylene might indicate that the hormone could complement the action of some effectors (Valls *et al.*, 2006). On the other hand, lectin genes are also controlled by HrpG and might therefore play a role in the process of attachment to plant roots (Valls *et al.*, 2006).

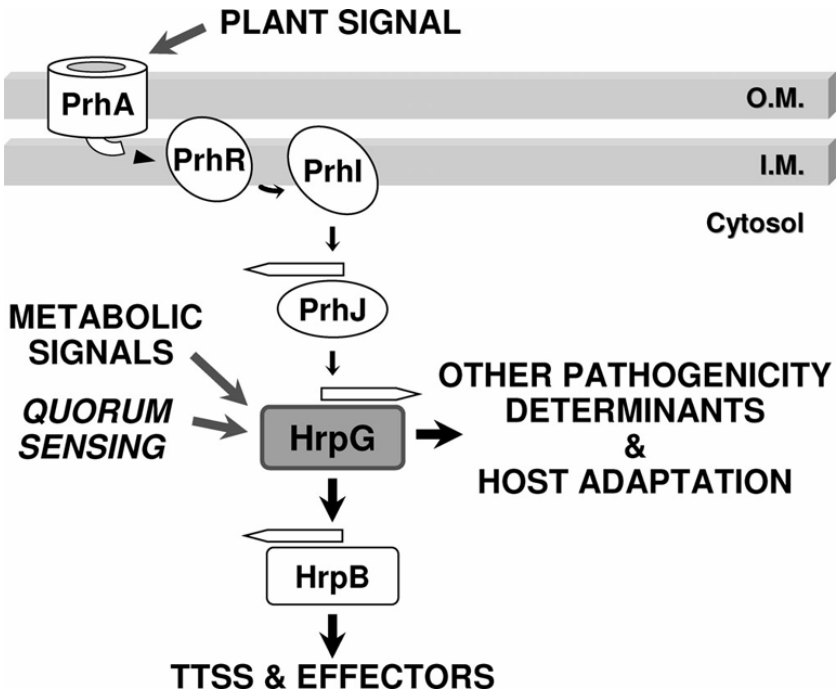


Figure 1.13. Hrp regulation cascade in *R. solanacearum*. Rounded forms symbolise proteins, and open arrows, gene sequences. [From Valls *et al.*, 2006].

1.2.7 Life cycle

R. solanacearum is a soil and water borne bacterium that enters the plant through the roots and invades the xylem vessels (Kelman, 1953). Systemic colonization requires secretion of cell wall-degrading enzymes and EPS, controlled by a regulatory network that uses a PhcA transcriptional regulator (Schell, 2000). After destroying the host plant, the bacterium is likely to return to a non-host environment where it is able to survive in soil, water or plant debris until contact with a new host (Denny *et al.*, 1994). Within plant tissues, high densities of the pathogen increase expression of multiple virulence genes, which are repressed by low bacterial densities in non-host environments (Schell, 2000; Bhatt & Denny, 2004).

1.2.7.1 Directed motility to the host

In the environment, *R. solanacearum* senses specific stimuli and moves toward plants to find more favourable conditions (Yao & Allen, 2006, 2007), making use of swimming motility (see section 1.2.2) for that (Tans-Kersten *et al.*, 2001). *R. solanacearum* was actively attracted by chemotaxis to diverse amino acids and organic acids, and especially to host root exudates, whereas those from a non-host were less attractive (Yao & Allen, 2006). Furthermore, the ability of the pathogen to locate and effectively interact with the host was significantly dependent on aerotaxis or energy taxis (Yao & Allen, 2007), which had been already described for *R. solanacearum* (Kelman & Hruschka, 1973). Aerotaxis guides bacteria toward optimal intracellular oxygen concentration (Taylor *et al.*, 1999). Thus, several *R. solanacearum* aerotaxis-deficient mutants were impaired in either localizing on host roots or moving up an oxygen gradient (Yao & Allen, 2007). Swimming motility, chemotaxis and aerotaxis seem to have a role in the early stages of host invasion (Tans-Kersten *et al.*, 2001; Yao & Allen, 2006, 2007).

1.2.7.2 Life within susceptible hosts

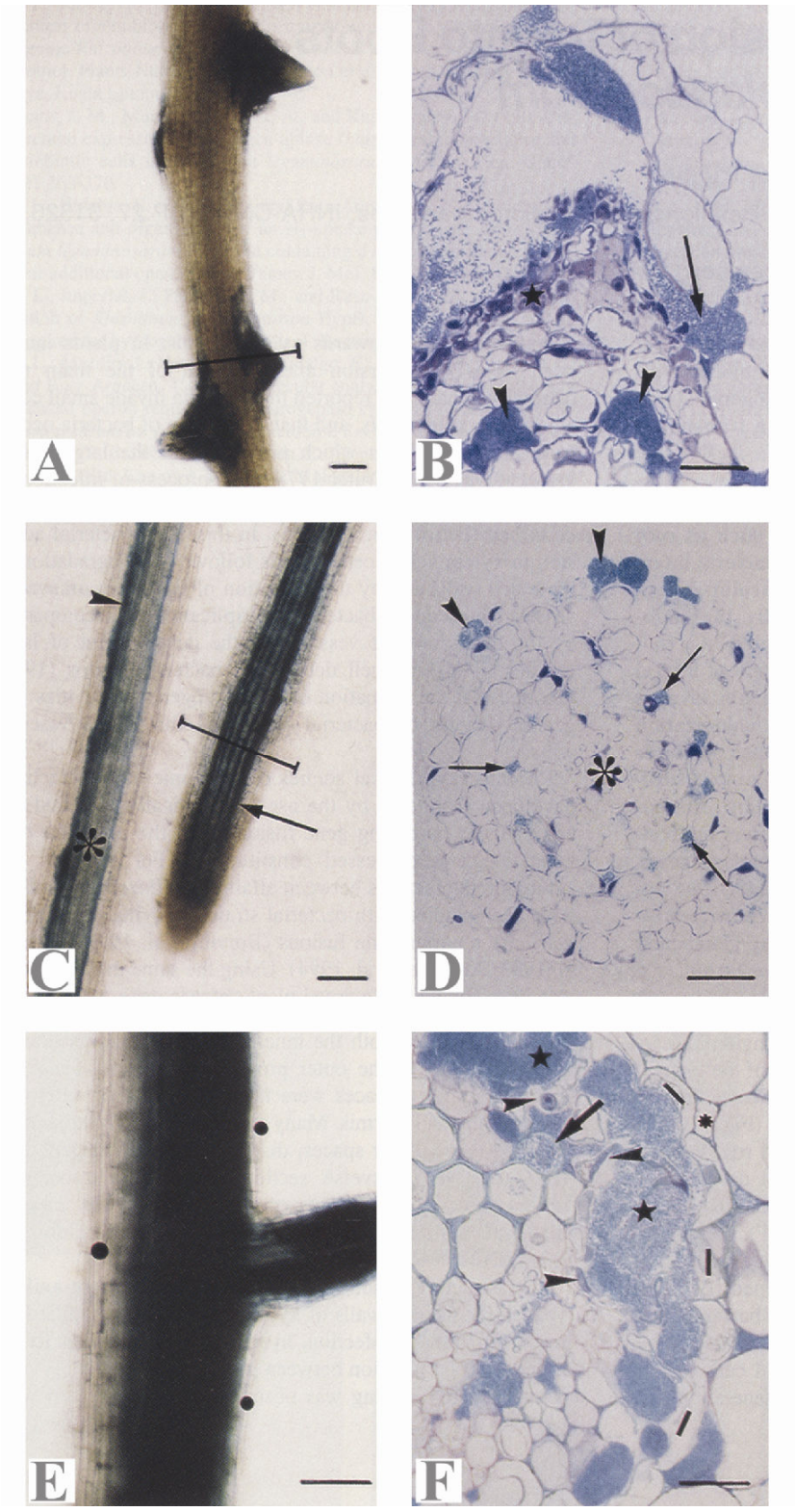
Once on the plant roots, *R. solanacearum* can enter the plant through physical wounds and/or natural openings (Kelman, 1953). In detailed

spatio-temporal descriptions of the *R. solanacearum*-host pathogenic interaction, Vasse *et al.* (1995, 2005) have distinguished three main stages.

The first of them is root colonization (Fig. 1.14). After localizing the host roots, *R. solanacearum* attaches at two precise root sites: root elongation zones and axils of emerging or developed lateral roots (Vasse *et al.*, 1995), probably due to the fact that the epidermal barrier is usually weaker in them. Moreover, root elongation zones are major sites of plant root exudation (Forster, 1986). In the attachment to roots, pili and/or LPS seem to have a role (Sequeira, 1985; Romantschuk, 1992), and more recently, the implication of flagella has also been demonstrated (Tans-Kersten *et al.*, 2001).

The second stage is the plant root cortical infection (Fig. 1.14) which starts at the sites previously colonized i.e. root extremities and axils of secondary roots. Due to the *R. solanacearum* infection, the root cortex of these zones has the intercellular spaces invaded and filled with bacteria (Vasse *et al.*, 1995). In the intercellular spaces the bacterium is likely to obtain nutrients from pectic polymers of the middle lamella by action of pectinolytic enzymes (Schell, 2000), and also folate concentration in the spaces seems to contribute to vigorous proliferation of the pathogen (Hikichi *et al.*, 2007).

Figure 1.14. Light microscopy of infection and colonization of tomato plants by *R. solanacearum* in longitudinal (A, C and E) and transversal (B, D and F) sections at (A and B) emergence sites of secondary roots; (C and D) tips of secondary roots; and (E and F) axils of secondary roots. (B) Infection in a cortical space (arrow); intercellular bacterial pockets (arrowheads); necrotic meristematic cells (black star). (C) Infection at the tip (arrow); vascular cylinder (asterisk); infection adjacent to the vascular cylinder (arrowhead). (D) Intercellular infection of inner cortex (arrows); colonization of epidermal cells (arrowheads); vascular cylinder (asterisk). (E) Uninfected tissues (black dots). (F) Endodermis (dashes); infection in intercellular spaces of the vascular cylinder (stars); degraded xylem parenchyma cells (arrowheads); invaded xylem vessel (arrow); intercellular spaces of the inner cortex (small asterisk). Scale bars = 100 μm (A, C and E) and 25 μm (B, D and F). [From Vasse *et al.*, 1995].


A
B
C
D
E
F

(continued from page 56)

Infection proceeds to the inner cortex level of primary roots, with bacteria forming large intercellular pockets, and cortical cells next to them displaying features of degeneration (Vasse *et al.*, 1995). As disease progresses, swimming motility may help the invasive cells go through the cortex (Tans-Kersten *et al.*, 2001).

The third stage of the interaction is characterized by vascular cylinder infection and xylem penetration (Fig. 1.14). Bacterial advance from cortex to vascular parenchyma implies crossing the endodermis, a cell layer with suberized walls and phenolic compounds, thought to be a barrier to vascular pathogens (Beckman, 1987; Vasse *et al.*, 1995). Therefore, to bypass the endodermis, the bacterium might reach the vascular cylinder at sites where this barrier is compromised. These zones would be the root extremities, where the endodermis is not fully differentiated, and the axils of secondary roots, where it is reoriented by the outgrowth and development of lateral roots, all of them root exudation sites preferably colonized by the pathogen (Vasse *et al.*, 1995). Once in the vascular cylinder (Fig. 1.15), *R. solanacearum* infects the intercellular spaces of vascular parenchyma adjacent to xylem vessels, which eventually are also invaded. The pathogen can then be observed breaking into and filling xylem vessels, with the surrounding parenchyma cells being highly degraded (Vasse *et al.*, 1995; Saile *et al.*, 1997) (Fig. 1.16).

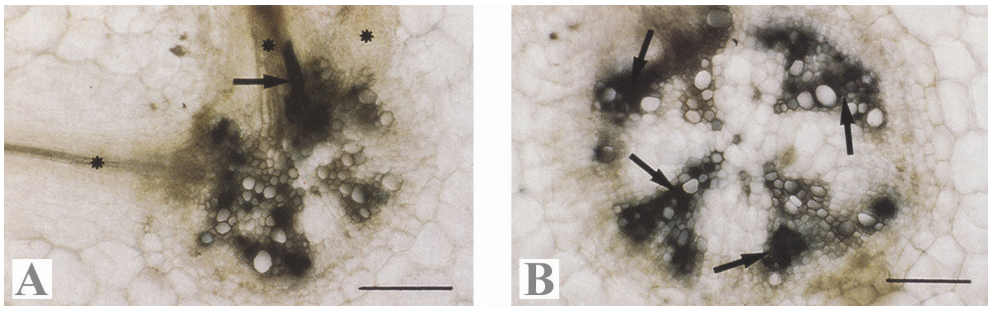


Figure 1.15. Vascular colonization by *R. solanacearum* of a partially wilted plant. (A) Upper part of the main root, an invaded xylem vessel (arrow) at the base of one of the three secondary roots (asterisks). (B) Collar zone, colonized vessels (arrows) in four vascular bundles. Scale bars = 250 μm. [From Vasse *et al.*, 1995].

Cell walls are destroyed by the hydrolytic enzymes secreted by *R. solanacearum* (see section 1.2.5.1). Within the xylem vessels, the pathogen moves throughout the stem to the upper parts of the plant while it is multiplying, being reported to reach even more than 10^{10} cells per cm of stem in tomato plants (Saile *et al.*, 1997; McGarvey *et al.*, 1999). It has been suggested that some *R. solanacearum* cells might form biofilms on host xylem vessel walls, which would protect them from host defenses (Tans-Kersten *et al.*, 2001) and could filter nutrients from the flow of xylem fluid (Yao & Allen, 2007). Although motility could help the pathogen spread out of infected vessels into adjacent uninfected ones, *R. solanacearum* is effectively non-motile in xylem vessels (Tans-Kersten *et al.*, 2001). Extensive multiplication and EPS production taking place in the water-conducting system lead to wilting of the host due to clogging of the vessels. The plant collapses and dies and *R. solanacearum* is released (Kelman & Sequeira, 1965) to a saprophytic life in the soil or other environments where it should survive until contact with a new host.

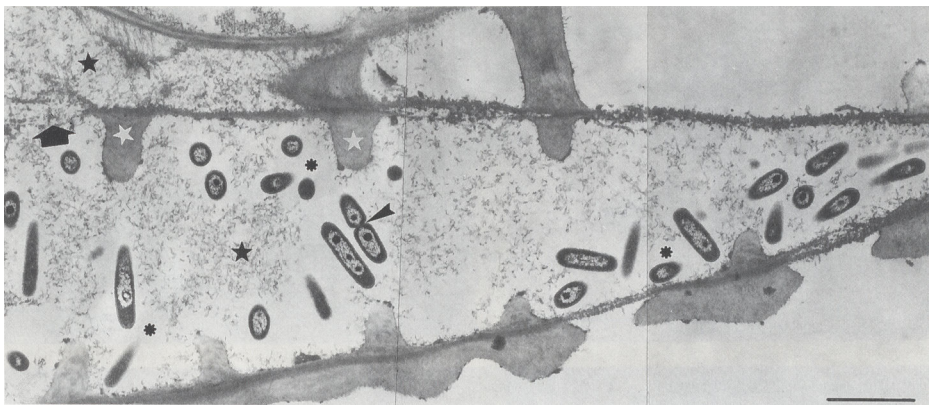


Figure 1.16. Electron microscopy of *R. solanacearum* penetration into xylem vessels. Degradation of a primary cell wall of an infected xylem vessel (large arrow); undegraded secondary cell wall appositions (white stars); bacterial cells are surrounded by an electronically empty halo (black asterisks); extracellular material (black stars); a dividing bacterium (arrowhead). Scale bar = 2 μm. [From Vasse *et al.*, 1995].

1.2.7.3 Life within resistant/tolerant hosts

Vasse *et al.* (2005) compared the root infection process by *R. solanacearum* in a susceptible tomato cultivar with that in a resistant one. They found a higher number of potential infection sites in the resistant cultivar due to a greater rhizogenesis, and two types of lateral roots which were differently colonized by a fully-virulent *R. solanacearum* strain: thick and short lateral roots with many long hairs, and thin and long lateral roots with few hairs. Thus, the thick short roots were colonized in the intercellular grooves at the surface, around the root base at lateral root emergence sites, and root extremities were infected in the apical meristem. No infection was observed in cortex or vascular tissues. In contrast, the thin long roots were less frequently colonized at the surface, and infection at lateral root emergence sites and root extremities was behind the apical meristem, namely in the intercellular spaces of the inner cortex, and only sometimes in protoxylem vesssels. In both types of lateral roots, a brownish coloration in zones proximal to those colonized by *R. solanacearum* was frequently observed, which might be due to the presence of compounds from secondary metabolism of polyphenols, probably responsible for the very limited root infection in the resistant cultivar (Vasse *et al.*, 2005). In fact, it was shown that increased phenols induced locally or systemically by a mycorrhizal fungus decreased *R. solanacearum* populations in the rhizosphere, on root surfaces and in the xylem of host roots (Zhu & Yao, 2004).

In less resistant or tolerant cultivars further movement of *R. solanacearum* onto xylem vessels of the stem can take place similarly to that in susceptible cultivars, with the difference that tolerant cultivars can limit the spread of the pathogen to the upper parts of the plant, probably by defence mechanisms such as inhibitor plant extracts, tyloses and gums that occlude vessels, and others, the quickness of activation by the plant being a key factor for successful resistance (Prior *et al.*, 1994).

1.2.7.4 Life in environments other than hosts

In the absence of a host, *R. solanacearum* is able to survive for variable periods of time in diverse natural habitats, where bacterial populations can be affected by both abiotic and biotic prevailing factors (van Veen *et al.*, 1997). The combination of them determines the fate of *R. solanacearum* in the environment.

Soil. Kelman (1953) referred to the ability of the bacterium to persist in agricultural soils devoid of susceptible crops for periods as long as ten (Roque, 1933), six (Wiehe, 1939) or four (Smith, 1944) years. In soils of temperate climates a range of work reports on *R. solanacearum* survival for variable periods of time (Dukes *et al.*, 1965; Graham *et al.*, 1979; Graham & Lloyd, 1979; Shamsuddin et *al.*, 1979; Devi *et al.*, 1981; Granada & Sequeira, 1983; Nesmith & Jenkins, 1983; Moffett & Wood, 1984; van Elsas *et al.*, 2000). As described, *R. solanacearum* withstood a four-year intercropping period keeping capacity of inducing wilting on hosts (Graham *et al.*, 1979). It could also be detected in bare fallow or weedy soil up to two years after crop removal (Shamsuddin *et al.*, 1979). Following field infestation, permissive soil temperatures and high moisture contents are major factors favouring *R. solanacearum* survival in the field (Kelman, 1953; Hayward, 1991). Moderate differences in soil moisture contents did not drastically affect soil-residing *R. solanacearum* populations, whereas severe drought resulted in rapid declines (van Elsas *et al.*, 2000, 2005). In fact, the bacterium seems to be poorly resistent to desiccation when surface layers of the soil are exposed to hot dry weather (Kelman, 1953). Wet and well-drained soils are more favourable for the pathogen than desiccated or flooded soils (Buddenhagen & Kelman, 1964; Hayward, 1991). Some soils seem to be suppressive to bacterial wilt (Hayward, 1991; French, 1994) due to antagonistic indigenous microbial populations (Nishiyama *et al.*, 1999). Persistence is likely to be enhanced in deeper soil layers, because of less temperature fluctuation, less grazing by protozoa or less competition by the indigenous microbiota (Graham & Lloyd, 1979). More recently, the bacterium survived up to one year in agricultural soil even after treatment with an herbicide to eliminate the hosts (van Elsas *et al.*, 2000, 2005).

Water. Early data on viability of *R. solanacearum* stored in sterile distilled or tap water under controlled conditions reported cells keeping culturability for more than 220 days, with higher numbers at 21°C than at 5°C (Kelman, 1956). Surprisingly, the pathogen readily multiplied during serial subcultures in pure water in the absence of energy sources (Wakimoto *et al.*, 1982) indicating a great ability to scavenge for scarce trace nutrients. As the majority of studies has been performed in artificial pure water, only few works report that *R. solanacearum* populations can survive in freshwater environments for variable periods of time (van Elsas *et al.*, 2001, 2005; Caruso *et al.*, 2005; Hong *et al.*, 2005) apparently withstanding the stresses imposed even under temperate climate conditions. However, little is known about the ecology of the bacterium in these habitats (Caruso *et al.*, 2005; Coutinho, 2005), specially in the presence of other organisms. The persistence of the pathogen in water was favoured at permissive, non-extreme temperatures and by the presence of sediment, probably because of physical protection of particulate matter offering a refuge; however, it was unfavoured in sediment subjected to drying and by levels of seawater salts similar to those of water in coastal areas, due to osmotic tensions (van Elsas *et al.*, 2001, 2005). In surface water layers, incident light in a light-dark regime had a detrimental effect on *R. solanacearum* survival, presumably due to either a direct effect on cells caused by photo-oxidative damage or an indirect effect by stimulating growth of algae which might be competitors or antagonists of the pathogen (van Elsas *et al.*, 2001, 2005). Long-term studies carried out on *R. solanacearum* population densities occurring in natural surface water used for field irrigation in Spain (Fig. 1.17) and the USA similarly indicated a cyclic effect of temperature variation on population sizes (Caruso *et al.*, 2005; Hong *et al.*, 2005). Thus, during spring and summer (at 14°C or higher) bacterial population levels were relatively high but, in autumn and winter (9°C or lower) recovery of bacterial cells was usually unsuccessful (Biosca *et al.*, 2005; Caruso *et al.*, 2005; Hong *et al.*, 2005). Furthermore, these naturally starved, cold-stressed cells were able to cause disease in host plants after inoculation, suggesting ability for survival of the pathogen under adverse conditions and pointing

out the role of water in bacterial wilt transmission, even in the colder months (Caruso *et al.*, 2005).

Figure 1.17. Water flowing in the river Tormes (Salamanca), where *R. solanacearum* has been detected in the last years in Spain. [Picture: B. Álvarez].

Plant-associated. Association of *R. solanacearum* with either reservoir plants or plant debris has been frequently suggested to promote survival of the pathogen in soil and water, and favour overwintering in temperate regions (Dukes *et al.*, 1965; Graham *et al.*, 1979; Devi *et al.*, 1981; Granada & Sequeira, 1983; Hayward, 1991; Wenneker *et al.*, 1999; van Elsas *et al.*, 2000). Thus, a number of solanaceous and non-solanaceous weeds have been found to harbour *R. solanacearum* cells, often as latent, asymptomatic infections (Tusiime *et al.*, 1998; Wenneker *et al.*, 1999; Pradhanang *et al.*, 2000; Janse *et al.*, 2004). Several weeds growing either during the winter or summer seasons were considered potential hosts of *R. solanacearum* (Pradhanang *et al.*, 2000), thus indicating possibility for persistence of the pathogen in the field during intercropping periods. In Europe, the most frequently reported carrier is bittersweet nightshade (*Solanum dulcamara*, a common perennial semi-aquatic weed inhabiting river banks), and in a lesser extent also black nightshade (*Solanum nigrum*) and stinging nettle (*Urtica dioica*) (Olsson, 1976; Hayward, 1991; Wenneker *et al.*, 1999). In *R. solanacearum*-contaminated waterways, bittersweet nightshade is continuously exposed to the pathogen, which may overcome harsh winter conditions relatively shielded within the roots and stems of this plant, and from there it could be eventually released into the water (Olsson, 1976; Hayward, 1991; Elphinstone *et al.*, 1998; Janse *et al.*, 1998; van

Elsas *et al.*, 2001). Although infesting populations of *R. solanacearum* liberated from symptomless infected weeds are not as high as those from wilted plants (Pradhanang *et al.*, 2000), the continuous flow of released free-living cells contributes to the persistence of the pathogen in the environment.

1.2.7.5 Forms of resistance in the environment

PC-type. The potential biological significance of PC has been subjected to considerable speculation (Poussier *et al.*, 2003). PC-type variants (Fig. 1.18) can be spontaneously obtained in high numbers by growing the bacterium in static liquid medium, that is, in oxygen-limiting conditions. As they are almost undetectable in comparable shaken cultures, they may have some selective advantage over the wild type, like a higher motility that can favour aerotaxis in conditions of oxidative stress for the bacterium (Kelman & Hruschka, 1973). A model was postulated on a reversible switching between wild type and PC-type occurring when bacteria move from host to non-host environments, respectively (Denny *et al.*, 1994). According to it, PC-type variants might be specifically adapted to endure harsh conditions in the absence of a host (Denny *et al.*, 1994). The reversion from the PC to the pathogenic form observed *in planta* (Poussier *et al.*, 2003) seems to confirm the existence of this phenotypic conversion/reversion cycle (Denny *et al.*, 1994; Poussier *et al.*, 2003; van Elsas *et al.*, 2005).

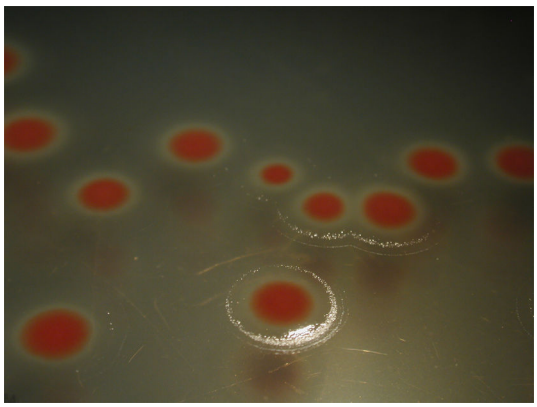


Figure 1.18. *R. solanacearum* PC-type colonies. [Picture: A. Trigalet].

Viable but non-culturable state. The ability of the bacterium to enter the viable but non culturable (VBNC) state may also be a factor influencing survival in harsh non-host environments principally bulk soil, soil-associated water or waterways. Since the state was first observed by Xu *et al.* (1982), it has been described for a great many bacterial species both pathogenic and non-pathogenic (Oliver, 2005), and also within the scope of plant-associated and plant pathogenic bacteria (van Elsas *et al.*, 2005). Bacterial cells in this state, although metabolically active (viable), are unable to grow on the media usually used for their culture (Oliver, 1993) and form dormant, non-proliferating cells. The state can be considered as a survival mechanism which some non-sporulating bacteria exhibit when environmental conditions become adverse (Roszak & Colwell, 1987; Grey & Steck, 2001; Oliver, 2005). Factors inducing the VBNC state vary according to bacterial species but always affect cellular growth (van Elsas *et al.*, 2005). Once favourable external conditions are restored VBNC cells may resuscitate from the non-culturable to the culturable state (Grey & Steck, 2001; Oliver, 2005), although this conversion is considered a conflicting matter (Oliver, 2005; van Elsas *et al.*, 2005), since it is usually difficult to prove that resuscitated cells actually come from VBNC forms and not from regrowth of a few remaining but undetected culturable cells (Oliver, 2005).

In *R. solanacearum*, a significant proportion of the population became VBNC in soil in less of a month by exposure of the cells at 4°C (van Elsas *et al.*, 2000) and within three days without dilucidating the inducing factor (Grey & Steck, 2001). The entrance in the VBNC state of the pathogen was observed to take place in liquid microcosms containing cupric sulfate, with the percentage of VBNC cells depending on copper concentration (Grey & Steck, 2001). Likewise, a temperature decrease to 4°C in pure water led to conversion of viable *R. solanacearum* cells to a state of non-culturability (van Elsas *et al.*, 2001; van Overbeek *et al.*, 2004). It was suggested that oxidative stress was involved in the effect of low temperature on *R. solanacearum* physiology, as the addition of catalase to the plating medium could partially restored colony forming ability (van Overbeek *et al.*, 2004). Cold-stressed *R. solanacearum* cells progressively lost capacity to wilt host plants while remaining infective although, eventually

within time, they even became unable to proliferate in the host, probably indicating severe cellular damage (van Overbeek *et al.*, 2004). The induction of the VBNC state in *R. solanacearum* has also been reported to occur *in planta* during infection (Grey & Steck, 2001). According to these authors, the proportion of cells becoming VBNC in the host increased after the plant underwent extensive necrosis. Moreover, as VBNC cells were detected in the upper parts of the plant before the culturable forms did, it was suggested that the VBNC cells would move through plant vascular system previously to the growing culturable forms (Grey & Steck, 2001). The same authors reported resuscitation of VBNC *R. solanacearum* in the presence of plant host rhizosphere. From VBNC cells in soil to which sterile seeds were added, culturable forms of the pathogen were observed only after plant germination and were found associated with the rhizosphere of both, symptomatic and asymptomatic plants (Grey & Steck, 2001). These authors further hypothesized that VBNC cells could have a role in the life cycle of *R. solanacearum* since, after collapsing the plant, the pathogen would arrive in the soil as a VBNC form allowing its survival until contact with a host rhizosphere, where it would be able to resuscitate, and infect plant roots anew (Grey & Steck, 2001).

Although the existence of a link between the PC and the VBNC forms of *R. solanacearum* would greatly contribute to the knowledge on the hidden saprophytic phase of the life cycle of this bacterium and its ecological role in the environment, the evidence of this link still remains as an unsolved question (van Elsas *et al.*, 2005).

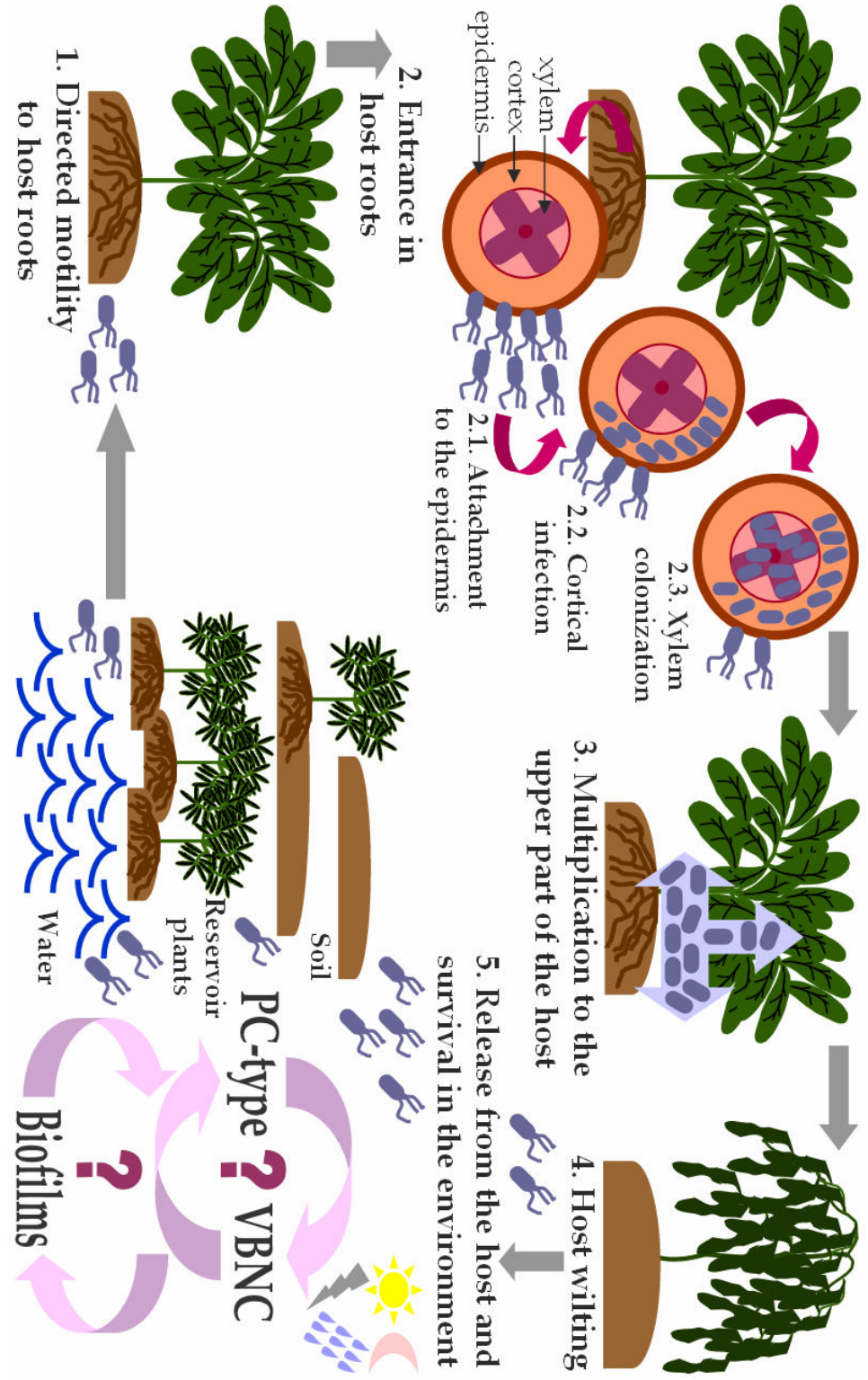
Biofilms. More recently, it has been suggested that biofilms may also have a role in *R. solanacearum* survival (Yao & Allen, 2007), as this kind of fixed communities of microorganisms adhered to each other by extracellular polymers help minimize fluctuations in environmental conditions (Morris & Monier, 2003). However, little is known on *R. solanacearum* capacity for biofilm formation and the factors involved in the process (Kang *et al.*, 2002). Similarly to some plant-associated bacteria, *R. solanacearum* forms biofilm-like structures in contact with abiotic and biotic surfaces. It was reported that different strains of the pathogen formed

biofilms on PVC wells at the liquid-air interface (Kang *et al.*, 2002; Yao & Allen, 2007), and also biofilm-like aggregations on the surface of tomato seedling roots (Yao & Allen, 2006). Moreover, aerotaxis might regulate biofilm formation, since aerotaxis-deficient mutants overproduced biofilms on abiotic surfaces (Yao & Allen, 2007). It was thus hypothesized that aerotaxis led cells to avoid the toxic effect of the higher oxygen concentrations of the liquid-air interface, and the thicker biofilms formed by cells trapped there had a protective function to facilitate survival (Yao & Allen, 2007).

A complete life cycle of *R. solanacearum* comprising the main phases mentioned in this section has been summarized in Fig. 1.19 (in the following page).

1.3 Prevention of bacterial wilt and control of *Ralstonia solanacearum* dissemination

The bacterial wilt diseases caused by members of the *R. solanacearum* species complex have never been more important (Allen, 2005). They produce severe crop losses with an economic impact worldwide (Allen, 2005; Elphinstone, 2005). Following introduction of race 3 biovar 2 in European potato fields and waterways (see section 1.1.3), the pathogen has been considered a quarantine organism by the EU (Anonymous, 2000) and the European and Mediterranean Plant Protection Organization (EPPO) (López & Biosca, 2005), which have developed specific legislation, protocols for detection, and measures to apply in case of an outbreak occurs (EPPO, 1990; Anonymous, 1998, 2006). In the United States, recent introduction of race 3 biovar 2 in ornamental plants (Swanson *et al.*, 2005) has made this pathogen be subject to strict regulations. Thus, it is considered a quarantine pathogen and a Select Agent in Agroterrorism Protection Act of 2002 (Lambert, 2002; Allen, 2005; Swanson *et al.*, 2005). All these legislations may have economic significance in developing countries exporting to the EU and the United States, since in many of them the pathogen is endemic (Allen, 2005).


1. Directed motility to host roots
2. Entrance in host roots
epidermis
cortex
xylem
2.1. Attachment to the epidermis
2.2. Cortical infection
2.3. Xylem colonization
3. Multiplication to the upper part of the host
4. Host wilting
5. Release from the host and survival in the environment
Water
Reservoir plants
Soil
PC-type ? VBNC
Biofilms ?

1.3.1 Prevention

Many measures have been recommended to prevent the appearance of bacterial wilt in a particular area, such as irrigation of crops with water proved to be non-contaminated with the pathogen or the use of *R. solanacearum*-tested free seeds (López & Biosca, 2005). In fact, EU Directives regulate the realization of annual surveys of surface water and potato seed (Anonymous, 1998, 2006). Likewise, ware potatoes imported from areas where the disease has been reported are tested (López & Biosca, 2005), since they are a major potential means of *R. solanacearum* introduction (French, 1994; Hayward *et al.*, 1998). Dissemination of the pathogen can also occur from exchange of contaminated plant material. This can be prevented by a previous sanitary control. Dissemination of the pathogen may also occur from exchange of machinery and/or workers from infested to uninfested fields (Hayward, 1991; French, 1994). External disinfection of material and of shoes can be recommended.

Accurate detection methods of high sensitivity and specificity are indispensable for designing efficient prevention strategies. In the EU several Directives indicate the compulsory methodology for analysis (Anonymous, 1998, 2006). In Spain, within the last ten years new serologic and molecular techniques have been developed in the Reference Laboratory for Phytopathogenic Bacteria to achieve a more rapid, specific and sensitive detection of *R. solanacearum* from different sources (Caruso *et al.*, 2000, 2002, 2003, 2005).

López and Biosca (2005) suggested some key points to be considered in prevention:

1.3.1.1 *R. solanacearum*-free soils

R. solanacearum can arrive in a soil via irrigation or surface runoff water and from infected plant tissues or infested machines used in agriculture (van Elsas *et al.*, 2000). After introduction, the pathogen can remain in the soil for many years, depending on soil moisture and temperature and the presence of plant debris, reservoir plants or hosts (see

section 1.2.7.4). Thus, volunteer potato plants should be removed from the field, as they can act as an inoculum source.

Detection of *R. solanacearum* in soil prior to plant cultivation is essential to prevent the disease in the field. The difficulties in succeeding may concern an irregular distribution of the pathogen due to the heterogeneous nature of soil, the lack of efficient soil sampling procedures, possible low population densities in soil and the presence of inhibitors, antagonists and competitors (López & Biosca, 2005), and also to the presence of this pathogen in the VBNC state (van Elsas *et al.*, 2000; Grey & Steck, 2001). As mentioned (see section 1.2.7.5), *R. solanacearum* under VBNC induction is no longer detectable by cultivation-based methods but it still remains viable and potencially pathogenic (Grey & Steck, 2001). In spite of the possibility of the existence of VBNC forms of the pathogen in nature, diverse techniques proved to be reliable for the detection of the bacterium (Pradhanang *et al.*, 2000; López & Biosca, 2005), such as dilution plating on a modified SMSA medium (Englebrecht, 1994; Elphinstone *et al.*, 1996), immunofluorescence colony staining (van Elsas *et al.*, 2000), enrichment-ELISA using specific monoclonal antibodies (Caruso *et al.*, 2000) or enrichment-PCR with specific primers (Pradhanang *et al.*, 2000).

1.3.1.2 *R. solanacearum*-free water

Water can be a major means of introduction and dissemination of *R. solanacearum* to crop fields. A number of bacterial wilt outbreaks in the EU member states were related to irrigation of potato fields with *R. solanacearum*-contaminated water diverted from natural waterways (Olsson, 1976; Hayward *et al.*, 1998; Palomo *et al.*, 2000; Caruso *et al.*, 2005). The sources of inoculum have been suggested to be rotten potatoes discarded by the growers and industrial or domestic effluents and residues from contaminated tubers imported from developing countries, where the disease is endemic (López & Biosca, 2005). For that reason, potato process water should be surveyed (Anonymous, 1998, 2006; Hayward *et al.*, 1998; Farag *et al.*, 1999). On the other hand, establishment of *R. solanacearum* in waterways of temperate zones is likely to depend on its capacity to cope with reduced nutrient availability and low temperatures (van Elsas *et al.*,

2001, 2005; van Overbeek *et al.*, 2004) but can be favoured by the presence of *Solanum dulcamara* (Elphinstone *et al.*, 1998a, b; Janse *et al.*, 1998).

Early detection of *R. solanacearum* in waterways or surface irrigation water is essential to prevent introduction to new areas (López & Biosca, 2005) (Fig. 1.20). In contaminated waterways, *R. solanacearum* can be easily detected in warm months even at low densities by direct plating on modified SMSA agar (Elphinstone *et al.*, 1996; Biosca *et al.*, 2005; Caruso *et al.*, 2005) whereas it is difficult to recover from cold waters (Biosca *et al.*, 2005; Caruso *et al.*, 2005), probably due to a VBNC induction which might reverse once favourable temperature conditions are restored (Grey & Steck, 2001).

Figure 1.20. Sampling in the river Tormes (Salamanca). [Pictures: J. L. Palomo].

Although recovery of low-temperature-induced VBNC cells is not always possible (van Overbeek *et al.*, 2004), techniques aimed to detect cold-stressed *R. solanacearum* have been developed. Caruso *et al*. (2005) compared two selective broths for enrichment of the bacterium from contaminated river water samples: modified Wilbrink (Caruso *et al.*, 2002) and modified SMSA, at two temperatures, 29°C and 35°C. They found that modified Wilbrink broth was more efficient at both temperatures in revovering cold-stressed *R. solanacearum* cells, even those non-culturable on solid media up to 25 days after their entry into the VBNC state. This enrichment proved better for double-antibody-sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA) detection of *R. solanacearum*, and combined with the most probable number (MPN) procedure (McCrady, 1915; Russek & Colwell, 1979) yielded the best results at 35°C

(Biosca *et al.*, 2005; Caruso *et al.*, 2005). The enrichment protocol can also be used prior to detection by PCR techniques (Seal *et al.*, 1993; Pastrik *et al.*, 2002), from which Co-operational PCR (Caruso *et al.*, 2003) can be specially recommended due to its high sensitiveness.

1.3.1.3 *R. solanacearum*-free seed potato

Latently infected but healthy-appearing tubers may harbour the pathogen on the surface, lenticels and vascular tissue (Hayward, 1991). These tubers, which can be obtained from tolerant or susceptible potato cultivars that have not developped bacterial wilt symptoms, are likely to disseminate the pathogen (Hayward, 1991; French, 1994). In the last decades, more frequent trade in seed potatoes has increased the need for disease monitoring (Wood & Breckenridge, 1998; Caruso *et al.*, 2002; López & Biosca, 2005). For that reason, EU Directives (Anonymous, 1998, 2006) have as an objective to limit the spread of the bacterium through latent infections by periodical inspections and analyses of imported stocks and home production of tubers (López & Biosca, 2005).

R. solanacearum-free seed potato producing methods should be adapted to the diverse conditions found in the different potato farms (López & Biosca, 2005). In that sense, farmers should be trained on the most convenient practices and treatments according to their available resources (Dhital *et al.*, 1996). Clean potato seeds can be produced by several methods either from non-contaminated mother tubers in a pathogen-free area or multiplied *in vitro* (Wood & Breckenridge, 1998; Kinyua *et al.*, 2005; Lemaga *et al.*, 2005; Mienie & Theron, 2005). All these methods, together with accurate detection techniques (Seal *et al.*, 1993; Anonymous, 1998, 2006; Elphinstone *et al.*, 2000; Weller *et al.*, 2000; Caruso *et al.*, 2002; Pastrik *et al.*, 2002) have successfully reduced the presence of *R. solanacearum* in seed potatoes (López & Biosca, 2005).

The concept "pathogen-free"

There is a need to comment on the concept "pathogen-free " that has just been presented in this section.

The positive detection of the pathogen of interest depends on the sensitivity of the method used that is about 10^3 cells/ml with the immunofluorescence, about 10^{1-2} cells/ml with the most accurate PCR-derived technique, and less than 10 cfu/ml by isolation.

The positive detection also depends on the sampling. Statistical analyses that are based on the Most Probable Number (MPN) and/or the Poisson's law will ascertain the positive presence with some reliability at a given level of confidence. For instance when the analysis of 200 potato seed tubers is negative, it should not be stated that this seed lot is free from the pathogen, but rather that this seed lot is not contaminated at a higher rate than 2.5% with a confidence level of 99%.

1.3.2 Control

Effective control requires an integrated management program adapted specifically to each zone, provided the diversity of bacterial wilt problems worldwide (French, 1994; López & Biosca, 2005).

An important aspect for successful control of *R. solanacearum* dissemination and bacterial wilt eradication is the realization of systematic surveillance on both, imported and home-produced plant material susceptible to the disease (Elphinstone, 2005). Surveys should be well-planified and have the sufficient resources to be carried out (López & Biosca, 2005). Growers should be trained for symptom detection and motivated to send the suspected samples to the laboratory. Since by EU requirements, infected material is bound to be incinerated, buried deep in places without risk of dissemination, processed for industry under official control, or used as animal feed after heat treatment (Anonymous, 1998, 2006), there is a need to compensate the growers when disease outbreaks are confirmed (López & Biosca, 2005).

1.3.2.1 Cultural practices

The level of bacterial wilt control by crop rotation is dependent on factors such as the ability of the local *R. solanacearum* strains to survive and maintain a remaining infective population in the absence of a host (Akiew & Trevorrow, 1994; Mariano *et al.*, 1998). Thus, only partial control of the disease can usually be achieved with short-term rotations. Intercropping of different time length with several plant species such as bean, cabbage, cowpea, onion, pea, or several cereals like maize or wheat has proved variable efficiency in bacterial wilt control (Devaux *et al.*, 1987; Hayward, 1991; Akiew & Trevorrow, 1994; French, 1994; Dhital *et al.*, 1996; Terblanche, 2002; Katafiire *et al.*, 2005; Lemaga *et al.*, 2005). Non-crops like marigold (*Tagetes patula*) combined with non-host winter crops reduced disease incidence on the subsequent host crop (Terblanche, 2002), presumably by production of thiophenes inhibiting *R. solanacearum* (Terblanche & de Villiers, 1997). To find suitable candidates for intercropping, and achieve a successful control of bacterial wilt by crop rotation systems, a methodology able to distinguish between host and non-host plants for *R. solanacearum* is needed (see Chapter 2.1).

Under extended bare fallow *R. solanacearum* populations may drop to undetectable levels (Buddenhagen & Kelman, 1964; Hayward, 1991; French, 1994; Pradhanang & Elphinstone, 1996; Stefanova, 1998), although the bacterium might survive in the deep moist soil layer for long (Hayward, 1991) or enter the VBNC state (van Elsas *et al.*, 2000; Grey & Steck, 2001). After an outbreak of bacterial wilt, EU Directives prohibit cultivation in the infested field for four years, and only non-hosts are allowed to be grown (Anonymous, 1998, 2006). An economically unprofitable but surely effective measure might be the recommendation of a bare fallow for 3-5 years in infested plots (López & Biosca, 2005).

Other changes in cultural practices to increase effectiveness of bacterial wilt control may reduce severity of the disease but not eradicate it (López & Biosca, 2005). For instance, potato crop losses were reduced after modification of planting date to seasons less conducive for the disease because of decreasing temperatures or increasing resting time for soil suppresive factors to act (Hayward, 1991; French, 1994; Mariano *et al.*,

1998). Addition of compost to soil enhanced decline rates of *R. solanacearum* populations and reduced numbers of diseased plants, while simulated solarisation did not significantly affect survival of the pathogen either in untreated or compost-amended field (Schönfeld *et al.*, 2003).

There are a great many weed species considered to be asymptomatic carriers of *R. solanacearum* and therefore with a role in persistence and dissemination of the pathogen in natural settings. In Europe, *S. dulcamara* is the most frequently reported (Elphinstone *et al.*, 1998; Elphinstone, 2005) but, the rhizosphere of several other weed species of *Solanum* and many species from other genera may also act as a shelter for cells of the pathogen (Hayward, 1991; Elphinstone, 2005; López & Biosca, 2005). Mechanical removal of *S. dulcamara* has been effective in control of bacterial wilt (Persson, 1998). For all weeds in general, herbicide treatment is recommended in eradication programs (López & Biosca, 2005).

With respect to irrigation water, disinfection by ozonization, addition of chrorine, hydrogen peroxide, and calcium hypochlorite have been shown to have some efficacy (Yamamoto *et al.*, 1990; Kelaniyangoda, 2002; Cariglia *et al.*, 2006), as well as percarbonic acid and peracetic acid with a catalase inhibitor (Niepold, 1999). A combined treatment of UV and chlorine in greenhouse watering systems has been recommended to clean up *R. solanacearum* (Cariglia *et al.*, 2006). As mentioned, in waterways, pathogen population decreasing can be eased by *S. dulcamara* removal (Persson, 1998).

1.3.2.2 Chemical control

Successful management of bacterial wilt by chemicals is difficult to achieve due to the fact that *R. solanacearum* localizes inside the plant xylem and is able to survive at depth in soil (Mariano *et al.*, 1998). Many products, mainly fungicides, antibiotics, soil fumigants and copper compounds have been used without complete success in *R. solanacearum* eradication (Hartman & Elphinstone, 1994). Moreover, antibiotics and other chemicals related to appearance of resistances in bacteria are not generally recommended. In spite of that, anti-microbial chemicals are still in use. For instance, the fumigants chloropicrin, 1,3-D or metam sodium, oxamyl or

ethroprop have been successfully tried (French, 1994; Weingartner & McSorley, 1994); under field conditions, methoxy ethyl mercuric chloride had an inhibitory effect against *R. solanacearum* and significantly reduced bacterial wilt incidence (Khan *et al.*, 1997); some products based on harpin or benzothiodiazol seem to have no effect *in vitro* but have been reported to enhance resistance in naturally resistant cultivars (Pradhanang *et al.*, 2002; López & Biosca, 2005).

1.3.2.3 Biological control

Alternative control strategies more acceptable to the environment have been described (Guo & Liang, 1985; Trigalet & Trigalet-Démery, 1990; Frey *et al.*, 1994; Trigalet *et al.*, 1994; Pradhanang *et al.*, 2002; Li *et al.*, 2006).

Bacterial antagonism against virulent strains of *R. solanacearum* has been extensively reported for bacterial species such as *Bacillus* sp. (Fucikovsky *et al.*, 1989; Anuratha & Gnanamanickam, 1990), *Burkholderia cepacia* (Aoki *et al.*, 1991), *Erwinia* sp. (Fucikovsky *et al.*, 1989), *Pseudomonas fluorescens* (Kempe & Sequeira, 1983; Ciampi-Panno *et al.*, 1989; Anuratha & Gnanamanickam, 1990), *P. glumae* (Furaya *et al.*, 1991) or *P. putida* (Priou *et al.*, 2006), isolated from soil and/or rhizosphere, and also for avirulent strains of the pathogen (Kempe & Sequeira, 1983; Trigalet & Trigalet-Démery, 1990; Hara & Ono, 1991; Frey *et al.*, 1994), generally used in pre-treatments of the host or in simultaneous inoculations (Trigalet *et al.*, 1994). However, in spite of being to some extent effective under controlled conditions, these biocontrol strategies often fail in natural environments (Trigalet *et al.*, 1994), probably due to a lack of profound knowledge on the underlying mechanisms, and on the pathogen ecology in different soils and root systems (López & Peñalver, 2006).

As endophytic antagonists, avirulent mutants of *R. solanacearum* apparently have the advantage of being adapted for survival *in planta* and able to compete directly with the pathogen (Trigalet *et al.*, 1994). However, spontaneous avirulent EPS-deficient mutants showed a limited systemic spread and declining populations in the host probably because of agglutination or binding to plant cell walls (Sequeira, 1982; Trigalet-Démery *et al.*, 1993; Trigalet *et al.*, 1994; Araud-Razou *et al.*, 1998). On the

other hand, it was reported that a weakly virulent mutant of *R. solanacearum* seemed to be more effective than a completely avirulent one (Hara & Ono, 1991). This can be due to the fact that the virulent mutant would be able to reach the xylem and activate the resistance response of the host (Trigalet *et al.*, 1994). Work concerning the use of *R. solanacearum hrp*-deficient mutants in biological control pointed out that root inoculation with the mutants prior to inoculation with a virulent strain limited bacterial wilt development even under optimal conditions for the disease, apparently by excluding the virulent strain from invading the stem (Trigalet & Trigalet-Démery, 1990), although in some cases the virulent strain was present at higher densities than the avirulent ones (Frey *et al.*, 1994). Moreover, in tomato plants, colonization by *hrp*-deficient mutants did not affect fruit production (Frey *et al.*, 1994). Notwithstanding, one of the inoculated virulent strains produced a bacteriocin that inhibited the *in vitro* growth of the avirulent mutants (Frey *et al.*, 1994). Therefore, apart from a simple competition for room in the stem, potential antagonistic effects of native, virulent strains towards the avirulent introduced mutants should also be taken into account (Trigalet *et al.*, 1994).

There are also reports on other biocontrol methods. For instance, *R. solanacearum* was inhibited by extracts of *Casuarina* sp. containing antibacterial components as flavonoids (Guo & Liang, 1985). In the field, reduced incidence of bacterial wilt was obtained with extracts of garlic pods (*Allium sativum*) and neem seeds (*Azaradicta indica*) (Singh, 1997). Likewise, other plant-derived extracts and essential oils reduced *R. solanacearum* populations in soil (Pradhanang *et al.*, 2002; Arthy *et al.*, 2005) or *in vitro* (Teixeira *et al.*, 2003). A bio-pesticide based on the activity of some unidentified metabolites produced by a strain of *Paenibacillus polymyxa*, effective against bacterial wilt and other plant diseases, has been successfully developed and patented (Li *et al.*, 2006).

A combination of several of the biocontrol agents available and/or their inclusion in an integrated control scheme may surely increase effectiveness against the bacterial wilt disease (López & Peñalver, 2006).

1.3.2.4 Resistant cultivars

Work has long been done with certain success to find wild plant species close to *R. solanacearum* hosts, which were naturally resistant to bacterial wilt (Kelman, 1953). Breeding and selection from crosses involving these species yielded some commercial cultivars or breeding lines with stable resistance to the disease, and with agronomic traits and output acceptable to the market (Akiew & Trevorrow, 1994). Modern techniques, such as tissue-culture-induced (somaclonal) variation and those concerning genetic engineering have been proved to complement traditional breeding for bacterial wilt resistance (Schmiediche *et al.*, 1988; Daub & Jenns, 1989). However, there are still some difficulties in obtaining such breeding lines (Mendoza, 1994), probably due to the great genetic variability of strains within the *R. solanacearum* species complex. Thus, many cultivars show unstable resistance (Hartman & Elphinstone, 1994), or those tolerant are not recommended, since they can be carriers of latent infections (Priou *et al.*, 1999). Therefore, field testing of resistant cultivars should be performed in different geographical locations to fulfil a standard evaluation procedure on the stability of the resistant genes under diverse conditions optimal to the disease (Akiew & Trevorrow, 1994). On the other hand, naturally occurring strains of the bacterium might overcome resistance and change towards a more virulent population. Molecular studies on genetic basis of *R. solanacearum* pathogenicity might help solving this inconvenience, being of great usefulness in the development of resistance to the pathogen (Akiew & Trevorrow, 1994).

1.3.2.5 Integrated control

It is generally assumed that eradication of the pathogen from protected production systems can be tackled with better perspectives than eradication from the environment (Elphinstone *et al.*, 2006). In spite of that, integrated strategies to control the disease on infested land can be very useful to allow farmers to obtain an economically acceptable crop production (Gildemacher *et al.*, 2006).

As healthy seed is indispensable (French, 1994), activities such as promoting the use of high-quality seed among farmers through

demonstration trials, acquainting farmers with high-quality seed production systems, and including a bacterial wilt test in seed certification programs would be crucial to manage the disease (Priou *et al.*, 2006). Potato brown rot successful integrated control package would involve adoption of sanitation measures such as seed certification, seed imports from pest free areas, routine analyses for detection of the pathogen in crops and irrigation water, crop rotation, targeted removal of volunteer potato plants and/or *S. dulcamara* when present, roguing of sick plants, organic and/or inorganic amendments, soil treatments and disinfestation of cultivation tools (French, 1994; Mariano *et al.*, 1998; Elphinstone *et al.*, 2006; Gildemacher *et al.*, 2006; Kinyua *et al.*, 2006; Priou *et al.*, 2006). Likewise, an integrated management program of bacterial wilt of tomato would include pre-plant soil fumigation, addition of plant activators and the use of cover crops (Momol *et al.*, 2006). For bacterial wilt of tobacco, integrated management normally consists of host resistance, crop rotation, soil fumigation and disinfectation during flower removal and leaf harvesting (Fortnum, 2006). The main factors to be considered in developing an integrated control strategy for bacterial wilt are summarized in Table 1.5 for races 1 and 3 but, they could be extended to the other races. French (1994) rated the factors with a value from 1 to 7; thus, when the sum of the factors considered in a particular place reached or surpassed 10, good control of the disease might be probable. A strategy should be site-specific, taking into account the prevailing socioeconomic factors influencing decision-making among local farmers (French, 1994).

Massive diffusion materials such as posters, leaflets, manuals, videos and/or radio announcements allow reaching more farmers with a message on prevention which facilitates adoption of bacterial wilt management technologies (Priou *et al.*, 2006). Raising the community awareness on the importance of training on bacterial wilt integrated management is imperative to prevent and control this devastating disease (Gildemacher *et al.*, 2006; Priou *et al.*, 2006).

Table 1.5. Main factors in management of the bacterial wilt disease. This table presents some factors to be weighted in developing a strategy for bacterial wilt control. Each factor was rated with a value from 1 to 7 by French (1994). A sum of 10 would usually be adequate for good control or even eradication. The efficiency in the combination of these factors depends on the geographical location, the crop of interest and the race of the pathogen present in the location. [Adapted from French, 1994].

BACTERIAL WILT INTEGRATED CONTROL STRATEGY		
FACTORS TO BE SELECTED	**IMPORTANCE FOR RACE 1**	**IMPORTANCE FOR RACE 3**
Amendments of soil	**1**	**1**
Biological techniques	**2**	**3**
Cold/temperate/ warm climate	**1**	**2**
Control of spread in water	**2**	**2**
Date of planting	**1**	**3**
Fumigants	**3**	**5**
Healthy seed	**3**	**3**
Host resistance/tolerance	**2**	**3**
Intercropping	**2**	**3**
Long/short rotation	**1**	**4**
Minimal cost	**2**	**1**
Moisture contents of soil	**3**	**2**
Nematode control	**4**	**2**
R. solanacearum-free soil/water	**7**	**7**
Removal of volunteers/reservoir plants	**2**	**4**
Roguing of wilted plants	**1**	**2**
Solarization	**1**	**1**
Suppressive soils	**2**	**4**

PART 2
EXPERIMENTAL CHAPTERS

CHAPTER 2.1

Comparative behaviour of *Ralstonia solanacearum* biovar 2 in diverse plant species

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Phytopathology **(2008) 98: 59-68**

Comparative behaviour of *Ralstonia solanacearum* biovar 2 in diverse plant species

Abstract

Ralstonia solanacearum causes bacterial wilt in numerous plant species worldwide. Although biovar 2 mostly affects solanaceous crops, identification of new hosts remains a matter of concern since there is still no clear-cut distinction between host and non-host plants. In this work we provide data based on histological studies on the status of 20 plant species, most of them of potential interest in crop rotation. Plants were watered with a β-glucuronidase-expressing derivative of *R. solanacearum* biovar 2 and, after a month of incubation, sections of roots and stems were analyzed to localize the pathogen on surface, in cortex and/or xylem. Depending on whether the xylem was colonized or not, plants were classified as hosts or non-hosts, respectively. Hosts generally affected in a few xylem vessels or occasionally in all xylem bundles were classified as tolerant**.** These included some cabbage, kidney bean and rutabaga cultivars, and the weed bittersweet nightshade (*Solanum dulcamara*). Non-hosts were the cultivars tested of alfalfa, barley, black radish, carrot, celery, colocynth, fennel, fiber flax, field bean, field pea, horseradish, maize and zucchini. However, barley and maize, though non-hosts, may act as reservoirs for the pathogen. The present work constitutes a basis for further studies on cropping systems in fields where *R. solanacearum* has been detected.

2.1.1 Introduction

There are few plant pathogenic bacteria that affect as many plant species as *R. solanacearum* (Kelman, 1953). Currently, its host range encompasses 53 botanical families (Hayward, 1994). This soil and water borne pathogen goes through the plant roots and colonizes the xylem causing the bacterial wilt disease, which progresses to death after vascular browning and necrosis (Kelman, 1953; Smith, 1920). The bacterium is widespread and produces severe economic losses worldwide (Elphinstone, 2005) as many of its hosts are of agricultural interest (Elphinstone, 2005; Hayward, 1994). The *R. solanacearum* species complex (Fegan & Prior, 2005) has traditionally been classified into races and biovars, according to host range and biochemical characteristics, respectively (Hayward, 1991).

In Europe, recent epidemics of potato brown rot (browning of potato vascular ring) caused by *R. solanacearum* race (R) 3 biovar (bv) 2 have triggered studies on the epidemiology of this pathogen**.** R3 bv2 is a group thought to be more adapted to solanaceous crops in temperate climates, though some non-solanaceous weeds are potential hosts (Hayward, 1994; López & Biosca, 2005; Tusiime *et al.*, 1998). Because *R. solanacearum* has been recognized as a quarantine organism in the European Union, very stringent control directives have been imposed (Anonymous, 1998, 2000, 2006). Notably, after an outbreak, there is a minimum four-year ban on planting host plants as well as regular checks for elimination of weed hosts and volunteer plants. In the United States, R3 bv2 has recently been introduced in ornamental plants, raising concern because of the threat posed to the potato industry (Swanson *et al.*, 2005). Strict security regulations are inflicted since, in the U.S., *R. solanacearum* R3 bv2 is considered to be a quarantine pathogen and a Select Agent of agroterrorism (Swanson *et al.*, 2005).

In the field, special attention has been paid to the identification of *R. solanacearum* hosts that could act as reservoirs during intercropping. For instance, *Solanum dulcamara*, a common semiaquatic weed inhabiting river banks in Europe, seems to be a shelter for this pathogen and favor its dissemination in river water used for irrigation, playing a role in the

persistence and spread of *R. solanacearum* in the environment (Elphinstone, 2005; Elphinstone *et al.*, 1998).

The host range of the *R. solanacearum* species complex is mainly based upon observation of wilt symptoms in the field, followed by isolation of the pathogen and further inoculation to a susceptible host plant to fulfil Koch's postulates. This approach has long been used to define true hosts of the pathogen as identified by Kelman (1953).

In rotation schemes carried out to prevent bacterial wilt, a number of candidates which were referred to as non-hosts often failed to eradicate the pathogen from fields infested with R3 bv 2 (Akiew & Trevorrow, 1994; French, 1994). This could possibly be explained by the lack of a clear-cut distinction between host and non-host plants.

Microscopy-based studies have been published describing different infection processes in susceptible and/or tolerant plants by the pathogen and its non-pathogenic mutants (Grimault & Prior, 1993; Vasse *et al.*, 1995, 2005; Araud-Razou *et al.*, 1998; Etchebar *et al.*, 1998). Furthermore, histological observations of root infection by the pathogen and further invasion of vascular bundles in the aerial part notably differ in susceptible and tolerant host plants (Grimault *et al.*, 1994; Rahman *et al.*, 1999). Because the host *vs*. non-host distinction may lead to conflicting results when applied to potential rotation crops, we believe that this status must be reexamined from a histological, microscopy-based approach of plant colonization by this pathogen.

In this work, a β-glucuronidase-expressing *R. solanacearum* bv 2 was used to determine the localizations of the pathogen in tissues from a series of potential rotation crop candidates. On the basis of light microscopy and bacterial isolation, these plant species were classified as hosts or non-hosts for the bacterium. Crops which were classified as non-hosts could be further assessed in open-field experiments to confirm their suitability for rotation schemes in areas where *R. solanacearum* R3 bv 2 had been detected.

2.1.2 Materials & Methods

2.1.2.1 Bacterial strains and growth conditions

The β-glucuronidase-expressing strain 1.3 derived from *R. solanacearum* strain IPO-1609 (R3 bv 2), called IPO-1609-Gus strain 1.3, was used in this study. Strain IPO-1609 was isolated by the Dutch Plant Protection Service from infected potato tubers cv. Bartina in 1995 (PD strain 2763) (van Elsas *et al.*, 2001). The *uidA* gene (Sharma & Signer, 1990) was introduced into the strain as described by Etchebar *et al.* (1998) for the *R. solanacearum* strain GMI 1000 (R1 bv 3), which was also included in some comparative assays, hereafter referred to as the β-glucuronidase-expressing GMI 1000 strain 9.2. Strains were kept at –80°C in a cryo-preservative medium containing 20% glycerol. After removal from the freezer, they were plated onto BGT agar (Boucher *et al.*, 1985) or Yeast Peptone Glucose Agar (YPGA) (Lelliot & Stead, 1987) and incubated at 30 °C for 3 days.

2.1.2.2 Preparation of inocula

Flasks containing BG liquid medium (Boucher *et al.*, 1985) were inoculated with the bacterial cultures and incubated for 24 h at 30 °C on a gyratory incubator shaker (185 r.p.m.). Bacterial suspensions were then adjusted to a concentration between 1.0-5.0 $x10^8$ CFU/ml. Inocula were all checked by plating onto BGT agar or YPGA to observe typical smooth colonies after a three-day incubation at 30°C. These suspensions were diluted in non-sterilized water for plant inoculations.

2.1.2.3 Preliminary assays to test the β-glucuronidase-expressing IPO-1609 strain 1.3

A suspension of the IPO-1609-Gus strain 1.3 was water inoculated in non-sterile peat mix with 15 five-week-old tomato plants (*Lycopersicon esculentum* cv. Roma) susceptible to *R. solanacearum* R3 bv 2. Watering was performed twice within a four-day interval with 150 ml/plant of a 10^7 CFU/ml suspension. Five uninoculated plants remained as negative

controls. Movement of the target bacteria was followed weekly for 35 days at low, middle and high stem levels, whose approximate distances from soil were 2-10 cm, 20-30 cm and 45-55 cm, respectively.

A similar assay with this derivative and the β-glucuronidase-expressing GMI 1000 strain 9.2 was also performed for comparative purposes on groups of six tomato plants cv. Supermarmande. All the assays were done in duplicate.

2.1.2.4 Evaluated species and growth conditions

Evaluated plants were: potato (*Solanum tuberosum* L. cvs. Désirée and Spunta) as positive controls, the aquatic weed bittersweet nightshade (*Solanum dulcamara*), and the crops: alfalfa (*Medicago sativa* cvs. Gea R1 and Symphonie), barley (*Hordeum vulgare* cv. Cork), black radish (*Raphanus sativus* cv. Gros Long d'Hiver), cabbage (*Brassica oleracea* cvs. Quintal d'Alsace and Virtudes), carrot (*Daucus carota* L. cvs. Nantaise and Tempo), celery (*Apium graveolens* cv. Istar), colocynth (*Citullus colocynthis* spp.), curly endive (*Cichorium endive* cv. Rida Doble de Verano), fennel (*Faeniculum vulgare* cv. Doux de Florence), fiber flax (*Linum* spp. cv. Hermès), field bean (*Vicia* spp. cv. Maya), field pea (*Pisum* spp. cv. Solara), forage cabbage (*Brassica* spp. cv. Proteor), horseradish (*Armoracia rusticana* spp.), kidney bean (*Phaseolus vulgaris* L. cv. Coco blanc), maize (*Zea mays* cv. Symphony), rutabaga (*Brassica napus nappobrassicus* cvs. Champion and Whilemsburger) and zucchini (*Cucurbita pepo* cv. Virginia).

Axenic potato seedlings were kindly provided by the French Federation for Potato (Paris). For bittersweet nightshade, seed sterilization was that described by Boucher *et al.* (1985). For preliminary assays of several species under axenic conditions, seeds were scarified by immersion in sulphuric acid (5 min for barley, fiber flax and maize, 3 min for carrot and fennel) with intermittent shaking, rinsed in sterile water and surface sterilized by soaking in sodium hypochlorite (3.6% of active chlorine) until seed coats were partially removed. Kidney bean seeds were immersed in sodium hypochlorite for 5 min. All seeds were washed three times with sterile water and were aseptically placed on Petri dishes containing BGT agar; they were protected from light by aluminium coating and kept in a

growth chamber at 24 ºC. Germinated seeds were transferred to sterile test tubes by deposition on either (i) stainless steel gauze at the surface of 65 ml of 0.43% MS (plant nutrient solution) liquid medium as previously described (Vasse *et al.*, 1995) for bittersweet nightshade, fennel and kidney bean, (ii) the surface of 35 ml of 0.5% soft agar containing 0.43% (wt/vol) of MS for barley, maize and potato, or (iii) the surface of 10g of either sterilized vermiculite (carrot) or vermiculite mixed with peat mix (fiber flax). The tubes were kept in a growth chamber (16 h light, 8 h dark; 24 ºC).

For experiments in non-axenic conditions, plants were grown in peat mix (66% black and white peat 7:3, 34% sand, pH 5.8, SC 8 Zaai, Vriezenveen Potgrond BV) in a greenhouse until plants reached 25-30 cm in height. They were then transferred to a growth chamber (16 h light, 26°C; 8 h dark, 22°C).

2.1.2.5 Plant inoculations

Small axenic plantlets (15-20 cm in height) were inoculated with the β-glucuronidase-expressing IPO-1609 strain 1.3 by aseptically adding to each test tube the appropriate volume (between 0.7 and 6.5 ml) from the suspension at 10^8 CFU/ml to reach a final concentration of 10^7 CFU/ml in either liquid solution, soft agar or vermiculite with/without peat mix. Volumes were added at the lowest part of the plant stem. For peat mix experiments, plants were inoculated with the strain by watering twice at a four-day interval with 150 ml/plant for all but one plant species and by soaking with 400 ml/plant for bittersweet nightshade. Soaking consisted of a temporary flooding of plant roots in the peat mix (about one week), until complete absorption had occurred. Most experiments were conducted with 18 individuals, and repetitions were carried out at least in duplicate. Inoculum concentrations were 10^7 CFU/ml and 10^6 CFU/ml respectively for watering and soaking inoculations. Uninoculated plants were used as negative controls.

At approximately one month postinoculation (mpi) each tested plant was cut at root and stem levels, and the peat mix in contact with roots was sampled to recover the bacterium, by the following procedures:

(i) Bacterial isolation, quantification and identification from plant segments. Stem segments analyzed were 2-3 cm and 4-5 cm long for axenic and non-axenic plants respectively, except for non-axenic alfalfa, carrot and fennel, from which 1-2 cm-length fragments were cut from the shoot-root transition region. Segments were all surface sterilized by immersion in a 70% ethanol solution for 10-20 sec and then by drying (axenic plants) or flaming (non-axenic plants). They were cut into small pieces, covered with sterile water and incubated at 30°C for 24 h. Aliquots were plated onto a modified semi-selective medium, South Africa (SMSA) (Elphinstone *et al.*, 1996) either by a Spiral Plate Maker (Interscience, Saint Nom la Breteche, France) or according to the surface spread plate technique, and incubated at 30°C for 3 days. Colonies were identified by PCR using primers OLI 1 and Y2 as described by (Seal *et al.*, 1993), phcA1 and phcA4 according to (Poussier *et al.*, 2003) or Cooperative PCR using primers OLI 1, OLI 2 and JE 2 as previously described (Caruso *et al.*, 2003).

Root segments analyzed to detect the presence of the pathogen on root surfaces were soaked in sterile water for 1 h at room temperature. From the macerating liquid the bacterium was isolated on SMSA (Poussier *et al.*, 2003) and indirect immunofluorescence (IF) was performed with a monoclonal antibody obtained against *R. solanacearum* (Araud-Razou *et al.*, 1998).

(ii) Bacterial isolation and identification from peat mix. An appropriate volume of sterile water was added to non-axenic peat mix in contact with plant roots, mixed and incubated without shaking at room temperature for 24 h. Aliquots were plated onto SMSA, and colonies were identified by PCR as mentioned above.

(iii) GUS activity assays. Histochemical assays for *uidA* expression were performed with the root and basal part of the stem. Root systems were immersed in a GUS reaction liquid solution (1.25 mM potassium ferricyanide 0.1 M; 1.25 mM potassium ferrocyanide 0.1 M; 0.25 mg/ml 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide 25 mg/ml; 12.5 mM KH_2PO_4-Na_2HPO_4 buffer 0.5 M pH 6.8), incubated at 30°C for 24 h, then kept at 4ºC in sodium cacodylate buffer 0.2 M adjusted to pH 7.2; for non-axenic plant roots, three previous rinses in sterile water, one in a 70% ethanol solution

and drying were required. The lower parts of stems were cut in longitudinal and thin transverse sections, and placed onto a 0.8% soft and sterile agar layer containing the GUS reaction solution as described elsewhere (Etchebar *et al.*, 1998); for non-axenic basal parts of stems, a previous short rinse in a 70% ethanol solution and drying were required. All covered sections were incubated at 30ºC for 24 h.

2.1.2.6 Microscopy methods

A Zeiss Axiophot microscope was used to screen root segments of interest and visualize thin longitudinal and transverse sections. Long blue segments (several cm) from the root system of all plants tested were observed in bright field microscopy without further staining. Representative colored parts (one to five) from these segments and occasionally from the basal stem zone were selected and cut into 4-5 mm pieces to be embedded in a 5% agar solution; after solidification, transverse sections of 150 μm were cut with a vibratome (Bio-Rad, Micro-cut H1200). Semi-thin root and basal stem sections (1-1.5 μm) were also obtained after Epson resin embedding with an ultramicrotome (Ultracut, Reichert-Jung) and observed by bright field microscopy after staining, as previously described (Vasse *et al.*, 1995). Pictures were taken with an adapted camera.

M3Z Wild Leitg and Leica MZ 7.5 stereoscopes were used to observe whole roots and sections of lower parts of stems. Color pictures were obtained with an adapted photo system and a digital camera Nikon Coolpix 990 placed on the device objective, respectively. Color pictures of the slides were taken either with a Nikon Coolpix 990 digital camera or an hp (Hewlett Packard) ScanJet 7400c scanner, at an image resolution of 600 ppi.

2.1.3 Results

2.1.3.1 Localization of the β-glucuronidase-expressing IPO-1609 strain 1.3 in tomato tissues

Histological localization and isolation revealed a heavy vascular infection of the basal and middle part of tomato stems by *R. solanacearum*

strain IPO-1609 expressing GUS in non-axenic peat mix-grown tomato plants cv. Roma (Table 2.1.1). Colonization by the pathogen was observed at 10 days postinoculation (dpi) at the collar level, and at 35 dpi blue color was found in the lowest part of the stems from all analyzed plants, in the middle part from seven out of 12, and in the higher part from two out of nine tested. Population sizes determined on SMSA at lower parts of stems ranged between 10^6 and 10^9 CFU/g dry weight (Table 2.1.1).

Table 2.1.1. Detection of the β-glucuronidase-expressing IPO-1609 strain 1.3 of *R. solanacearum* R3 bv 2 in tomato plants cv. Roma at 35 days postinoculation.

Plant number	Histological localization in stem (xylem)			Isolation from stem on SMSA (CFU/g dry weight)		
	Lower (2-5 cm)[a]	Middle (20-25 cm)	Upper (45-50 cm)	Lower (5-10 cm)	Middle (25-30 cm)	Upper (50-55 cm)
1	++[b]	+[c]	+	$7.6x10^9$	$>1.4x10^7$	$>5.6x10^7$
2	++	+	-[d]	$3.1x10^9$	$>1.4x10^6$	-
3	+	-	-	$2.5x10^7$	-	-
4	+	-	-	$>1.2x10^7$	-	-
5	++	+	-	$4.6x10^9$	$>1.4x10^6$	-
6	++	++	nt[e]	$7.7x10^9$	$6.8x10^9$	nt
7	+	-	-	$>1.2x10^7$	$5.7x10^3$	-
8	+	-	-	$>1.2x10^7$	-	-
9	+	-	-	$>1.2x10^6$	-	-
10	+	+	+	$7.5x10^9$	$>1.4x10^7$	$>5.6x10^6$
11	++	++	nt	$4.8x10^9$	$1.5x10^9$	nt
12	+	++	nt	$>1.2x10^7$	$6.0x10^9$	nt
Control	-	-	-	-	-	-

[a] Approximate height from soil level to the analyzed stem fragment.

[b] 1-4 xylem bundles stained.

[c] 1-10 groups of few vessels of xylem stained.

[d] Undetected.

[e] Not tested.

All inoculated tomato plants were positive for the isolation of the bacterium and *uidA* expression was found in a majority of xylem bundles in each individual tomato plant. Although β-glucuronidase assays using X-Gluc are not quantitative, an estimated limit of sensitivity of the Gus stain (based on detection of bacteria by dilution plating in tomato plants) would be around 10^4 CFU/g dry weight.

Comparative invasiveness between R. solanacearum R3 bv2 and R1 bv3 in tomato tissues. All non-axenic peat mix-grown tomato plants cv. Supermarmande watered with a suspension of the β-glucuronidase-expressing GMI 1000 strain 9.2 (R1 bv3) were completely wilted at six dpi, whereas the plants inoculated with IPO-1609-Gus strain 1.3 (R3 bv2) started to show disease symptoms at eight dpi. Localization of bacteria at collar level at this time revealed that the GMI 1000 derivative had strongly colonized the four bundles of xylem in all tested plants (Fig. 2.1.1 A), while comparably the β-glucuronidase-expressing IPO-1609 was not detected in all bundles of wilted plants (Fig. 2.1.1 B).

2.1.3.2 Localization of the β-glucuronidase-expressing IPO-1609 strain 1.3 in potato tissues

Colonization by the pathogen was first assessed in potato plants cvs. Désirée and Spunta under axenic conditions in soft agar. In cv. Désirée, at one mpi inoculated bacteria had entered through the axils of lateral emerging roots and extremities of stolons (Fig. 2.1.1 C). Blue staining in xylem vessels was localized at the collar level in five out of six analyzed plants. The invaded vessels mostly belonged to the same xylem bundle out of the four commonly present in potato stems, and one out of six plants showed the inner cortex colonized next to the invaded vessels (not shown). In cv. Spunta, at one mpi the bacterium was located in the root system at root extremities and lateral root emerging sites, and by two mpi a majority of xylem vessels were invaded, as shown in Fig. 2.1.1 D. Xylem vessels partially or even totally filled with bacterial cells were also observed (Fig. 2.1.1 E). For both cvs., most of the plants remained symptomless during the postinoculation period (one mpi for Désirée, two mpi for Spunta), with

some of them showing partial wilting. Under non-axenic conditions, peat mix-grown potato plants cv. Désirée started to show symptoms after 5 weeks postinoculation and by two mpi were already wilted. They had the central cylinders of primary and secondary roots heavily colonized at two mpi. In all potato tubers, eyes, heel ends and stolons were also strongly invaded (Fig. 2.1.1 F and G), and thick bacterial ooze was observed in vascular rings. The route by which the pathogen can penetrate the vascular tissues of tuber via the eye from outside is clearly illustrated in Fig. 2.1.1 F. IPO-1609-Gus also concentrated at the stolon/tuber junction in almost all tubers tested (not shown).

2.1.3.3 Localization of the β-glucuronidase-expressing IPO-1609 strain 1.3 in bittersweet nightshade tissues

Transverse sections of collars of bittersweet nightshade plants grown under axenic conditions in MS liquid solution, showed presence of bacteria in small groups of xylem vessels scattered throughout the bundles in all eight plants processed (Fig. 2.1.1 H). Presence of bacterial cells in these vessels was further confirmed by microscopic visualization of transverse semithin sections, which also enabled us to observe tyloses in vascular tissues (not shown). Likewise, observation of longitudinal and transverse semithin sections of roots revealed the presence of bacterial cells in the cortex and xylem areas (Fig. 2.1.1 I and J), but isolation of the β-glucuronidase-expressing IPO-1609 from stems was negative in all analyzed individuals. From non-axenic peat mix-grown plants, the collar sections embedded in the GUS soft agar displayed an *uidA* expression in the xylem vessels in three out of the 12 exposed plants. The bacterium was isolated from the stem of two out of three plants, accounting for $9x10^5$ CFU/g dry weight.

2.1.3.4 Localization of the β-glucuronidase-expressing IPO-1609 strain 1.3 in other plant species

Alfalfa. For cv. Gea R1 in the non-axenic peat mix-grown plants, there was no GUS activity detected inside plant tissues (Fig. 2.1.1 K). Bacterial isolations from the shoot-root transition region were also negative (Table 2.1.2). For cv. Symphonie in the non-axenic peat mix-grown plants, no blue staining was observed in either cortex or xylem and isolations were negative, although bacteria were detected on root surfaces (Table 2.1.2).

Barley. For cv. Cork under axenic conditions in soft agar, three out of the ten inoculated barley plants in two separate assays contained *R. solanacearum* (data not shown). Preliminary experiments were also performed in soft agar by flooding the lowest part of plant stems with a 10^8 CFU/ml inoculum. Transverse sections from these flooded stems displayed intense blue staining between the leaves forming the sheath of the plant and isolations of the bacterium from these flooded stems were frequently positive (data not shown). From the non-axenic peat mix-grown plants, transverse sections from all upper parts of root systems and basal part of stems showed a blue staining on the surface, but never in the cortex or xylem (Fig. 2.1.1 L). Isolations from stems were mostly negative, except for a few individuals, for which positive isolation could be correlated with external presence of the IPO-1609-Gus in the lower parts of the stem (Table 2.1.2).

Bean (field bean). For cv. Maya in non-axenic peat mix-grown plants, the labelled bacterium was detected on the root surface in 11 out of 36 inoculated plants. The cortex area was infected in less of half of these individuals, but this invasion was not observed in upper levels of the plant, whereas xylem tissues were never colonized by the IPO-1609-Gus. Isolations from plant stems correlated with the internal presence of the pathogen in the plant cortex (Table 2.1.2).

Table 2.1.2. Detection of the β-glucuronidase-expressing IPO-1609 strain 1.3 of *R. solanacearum* R3 bv 2 in plants grown in non-axenic peat mix.

Plant species and cultivar	Histological localization[a]					Isolation[b]	Plant status
	Roots			Lower part of stem			
	S[c]	C	X	C	X	Stem	
Alfalfa cv. Gea R1	0/19 0/23	nt nt	nt nt	0/19 0/23	0/19 0/23	0/19 0/23	Non host
Alfalfa cv. Symphonie	6/18 8/18	0/18 0/18	0/18 0/18	0/18 0/18	0/18 0/18	0/18 0/18	Non host
Barley cv. Cork	0/15 nt/+[e] 0/18 3/18	nt[d] nt 0/18 0/18	nt nt 0/18 0/18	0/15 0/17 0/18 0/18	0/15 0/17 0/18 0/18	0/15 0/17 0/18 3/18	Non host
Bean (Field bean cv. Maya)	6/18 5/18	3/18 2/18	0/18 0/18	0/18 0/18	0/18 0/18	3/18 2/18	Non host
Bean (Kidney bean cv. Coco blanc)	0/18 0/20	0/18 nt	0/18 nt	0/18 1/20	0/18 1/20	0/18 1/20	Tolerant host
Black radish cv. Gros Long d'Hiver	2/18 2/18	2/18 2/18	0/18 0/18	0/18 0/18	0/18 0/18	2/18 2/18	Non host
Cabbage cv. Quintal d'Alsace	18/18 18/18	0/18 0/18	0/18 0/18	12/18 15/18	12/18 15/18	18/18 18/18	Susceptible host

Cabbage	nt/ +	0/18	0/18	1/18	3/18	0/18	
cv.	nt	0/18	0/18	0/18	0/18	0/18	Tolera
Virtudes	0/18	0/18	0/18	0/18	0/18	0/18	nt host
	0/18	0/18	0/18	0/18	0/18	0/18	
Cabbage (Forage							Suscep
cabbage cv.	18/18	18/18	18/18	18/18	18/18	18/18	tible
Proteor)	18/18	18/18	18/18	18/18	18/18	18/18	host
Carrot cv.	nt/ +	0/13	0/13	0/13	0/13	0/13	Non
Nantaise	0/11	0/11	0/11	0/11	0/11	0/11	host
Carrot cv.	0/18	0/18	0/18	0/18	0/18	0/18	Non
Tempo	0/18	0/18	0/18	0/18	0/18	0/18	host
Celery cv.	nt/ +	0/20	0/20	0/20	0/20	0/20	
Istar	0/13	nt	nt	nt	nt	0/13	Non
	0/18	nt	nt	0/6	0/6	0/18	host
	0/18	0/18	0/18	0/18	0/18	0/18	
	0/18	0/18	0/18	0/18	0/18	0/18	
Colocynth	0/18	0/18	0/18	0/18	0/18	0/18	Non
	0/18	0/18	0/18	0/18	0/18	0/18	host
Curly endive cv.							
Rida Doble	18/18	12/18	0/18	9/18	9/18	18/18	Suscep
de Verano	18/18	12/18	0/18	9/18	9/18	18/18	tible
							host
Fennel cv.							
Doux de	nt/ +	0/13	0/13	0/13	0/13	0/13	Non
Florence	0/10	0/10	0/10	0/10	0/10	0/10	host
Fiber flax	0/23	nt	nt	0/23	0/23	0/23	
cv.	nt/ +	nt	nt	0/23	0/23	0/23	Non

Hermès	**2/18**	**0/18**	**0/18**	**0/18**	**0/18**	**2/18**	**host**
	2/18	**0/18**	**0/18**	**0/18**	**0/18**	**2/18**	
Field pea							
cv.	**6/18**	**2/18**	**0/18**	**0/18**	**0/18**	**2/18**	**Non**
Solara	**5/18**	**1/18**	**0/18**	**0/18**	**0/18**	**1/18**	**host**
Horseradish	**3/18**	**1/18**	**0/18**	**0/18**	**0/18**	**3/18**	**Non**
	3/18	**0/18**	**0/18**	**0/18**	**0/18**	**3/18**	**host**
Maize cv.	**nt**	**0/17**	**0/17**	**0/17**	**0/17**	**0/17**	
Symphony	**nt**	**0/18**	**0/18**	**0/18**	**0/18**	**0/18**	**Non**
	nt/ +	**0/20**	**0/20**	**0/20**	**0/20**	**1/20**	**host**
Rutabaga							
cv.	**12/18**	**12/18**	**3/18**	**0/18**	**3/18**	**nt**	**Tolera**
Champion	**12/18**	**12/18**	**3/18**	**0/18**	**3/18**	**nt**	**nt host**
Rutabaga							
cv. Whilems	**12/18**	**12/18**	**1/18**	**0/18**	**1/18**	**0/18**	**Tolera**
burger	**12/18**	**12/18**	**2/18**	**0/18**	**2/18**	**0/18**	**nt host**
Zucchini							
cv.	**1/18**	**0/18**	**0/18**	**0/18**	**0/18**	**1/18**	**Non**
Virginia	**2/18**	**0/18**	**0/18**	**0/18**	**0/18**	**2/18**	**host**

Results from different assays are indicated in different lines for each species and cultivar. S = surface, C = cortex, X = xylem.

[a] Number of plants with *R. solanacearum* detected by *uidA* expression/number of analyzed plants.

[b] Number of plants with *R. solanacearum* isolated from middle part of stems/number of analyzed plants.

[c] Number of plants with *R. solanacearum* detected by IF on root surface/number of analyzed plants.

[d] Not tested.

[e] Not tested on roots but positive in peat mix, as determined by isolation on SMSA and PCR of the colonies.

(continued from page 97)

Bean (kidney bean). For cv. Coco blanc, under axenic conditions in MS liquid solution, almost all transverse sections from roots showed a specific blue staining in vascular parenchyma. At the collar level, intense GUS activity was observed in the cortex and in xylem vessels all around the vascular ring in ten out of the inoculated 14 plants (Fig. 2.1.1 M), and the bacterium was isolated from stems in 12 of them (data not shown). From the non-axenic peat mix-grown plants, transverse sections embedded in GUS reactive agar revealed blue staining in xylem vessels, and isolation was positive from one out of the 38 inoculated plants, accounting for $1.1x10^2$ CFU/g dry weight.

Black radish. For cv. Gros Long d'Hiver, in the non-axenic peat mix-grown plants, the labelled bacterium was present on the root surface and in the cortex at root level for four of 36 plants, but was not detected in the majority. No blue staining was observed in the cortex at the lower stem level, or in the xylem regardless of the plant level. Isolations were positive only from the four plants (Table 2.1.2).

Cabbage. For cv. Quintal d'Alsace, from the non-axenic peat mix-grown plants, the pathogen was always found on root surface in all plants. No blue staining was observed neither in the cortex nor in the xylem at root level, in contrast to the lower part of stem, which was stained in the majority of the individuals. Isolations from these stems were all positive (Table 2.1.2). For cv. Virtudes, from the non-axenic peat mix-grown plants, *uidA*-expression was observed in some xylem vessels of the lower part of the stem of three individuals out of the 72 plants analyzed (Fig. 2.1.1 N). The presence of the bacterium was further confirmed in xylem vessels from semi-thin transverse sections (Fig. 2.1.1 O). Isolations from stem segments were negative in all analyzed plants (Table 2.1.2).

Cabbage (forage cabbage). For cv. Proteor, from non-axenic peat mix-grown plants, all the 36 inoculated individuals showed presence of the β-glucuronidase-expressing derivative on root surfaces and inner plant tissues i.e. cortex and xylem at root and lower stem levels. Isolations from stems were positive in all plants tested (Table 2.1.2).

Carrot. For cv. Nantaise, under axenic conditions in vermiculite, the analyzed plants did not develop a taproot system and GUS analysis of the small roots for the inoculated nine plants only showed adsorption on the surface in a limited number of root tips and some extremities of recently emerged secondary roots, which revealed the presence of the pathogen in cortical tissues (data not shown). In the lower part of the stems, no colonization of cortex or xylem was ever observed. From the non-axenic peat mix-grown plants, blue staining was only noticed on the epidermis of the shoot-root transition region, tuberous root and emerging lateral roots (Fig. 2.1.1 P). Isolations were negative in all the tested plants (Table 2.1.2). The taproots collected from inoculated plants did not differ in size with respect to those from the uninoculated plants. For cv. Tempo, from non-axenic peat mix-grown plants, no specific blue staining was ever observed in inner or outer plant tissues, in all the 36 analyzed plants (Table 2.1.2).

Celery. For cv. Istar, in non-axenic peat mix-grown plants, there was no *uidA* expression and isolations were negative from the outer and inner tissues of all 87 tested plants (Table 2.1.2).

Colocynth. In non-axenic peat mix-grown plants, no blue staining was ever observed in tissues of plant sections and isolations were negative for the 36 inoculated plants (Table 2.1.2).

Curly endive. For cv. Rida Doble de Verano, in non-axenic peat mix-grown plants, there was a superficial adsorption on the root systems by the IPO-1609-Gus in all inoculated plants. The roots were frequently invaded in the cortical tissues, although the bacterium was not detected in the xylem. At the lowest stem level, a localized blue staining appeared in both, cortex and xylem, for 18 out of 36 plants. Isolations were positive from the stem of all the 36 individuals (Table 2.1.2).

Fennel. For cv. Doux de Florence, under axenic conditions in MS liquid solution, longitudinal and transverse sections of the whole small root system of the nine inoculated plants revealed the presence of *uidA*-expressing bacteria in the pericycle and cortex areas, respectively (data not shown). All isolations were negative from the shoot-root transition region. From the 23 non-axenic peat mix-grown plants, no *uidA* expression in the inner plant tissues was observed (Fig. 2.1.1 Q).

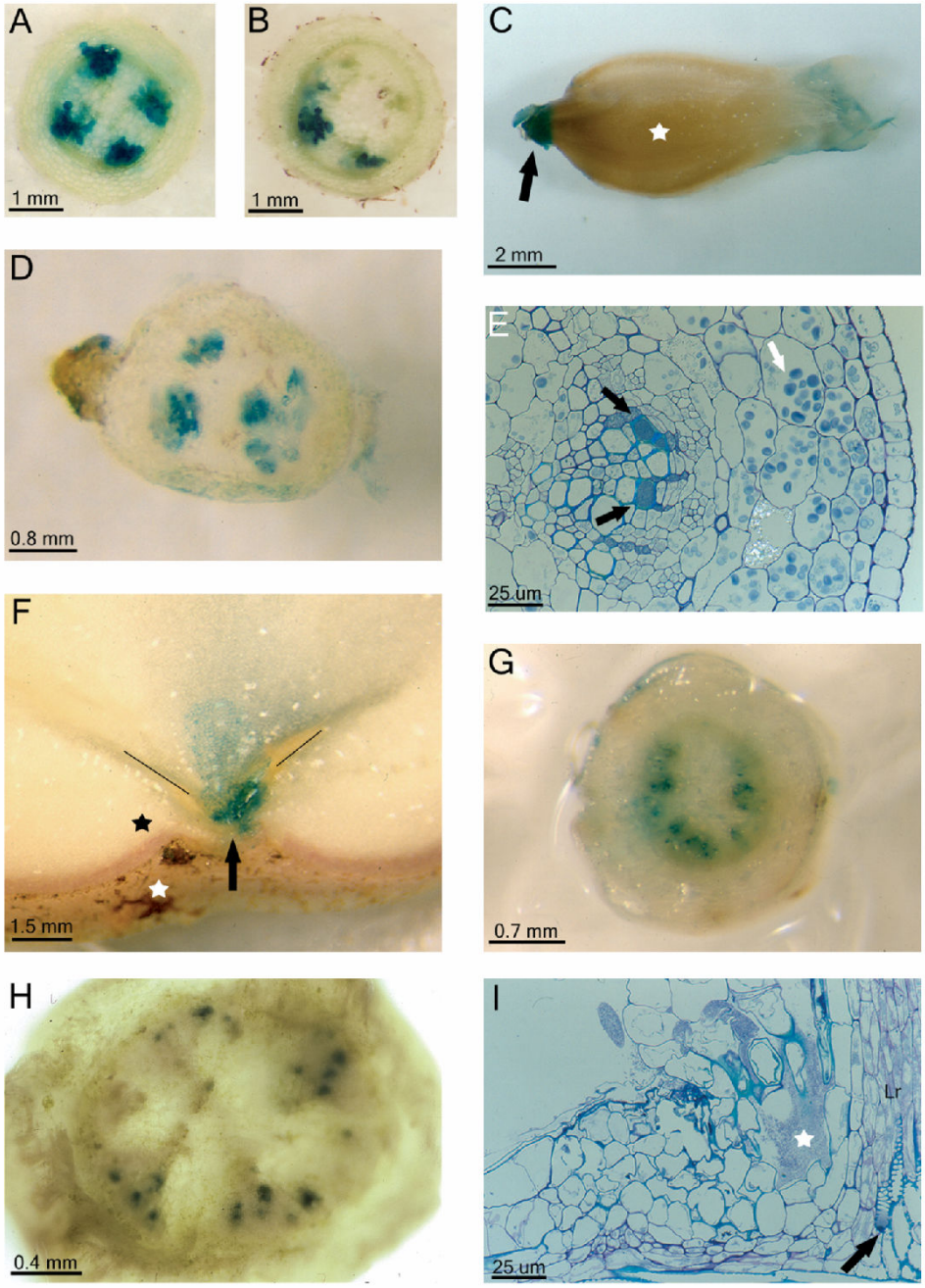
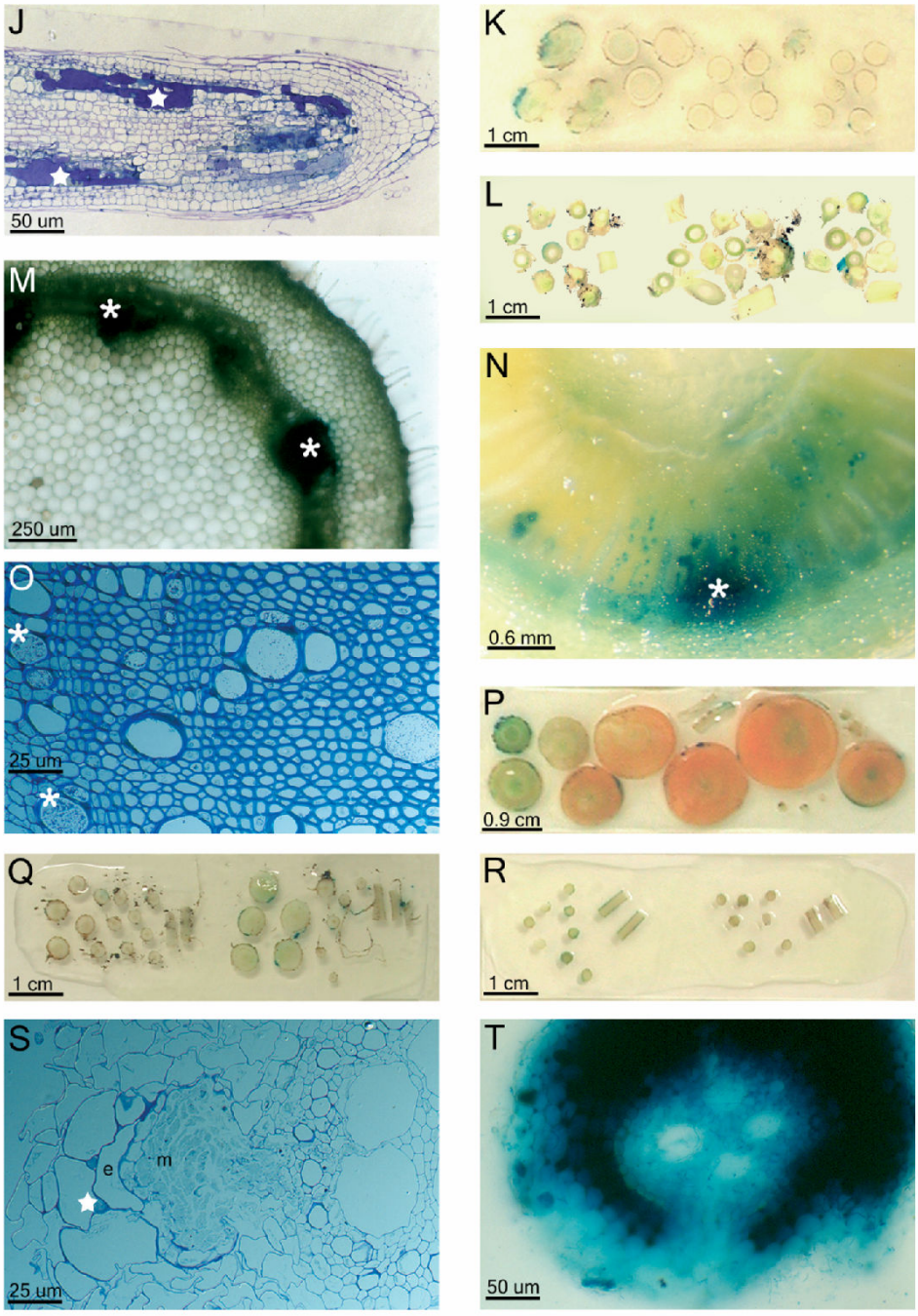


Figure 2.1.1. Light microscopy of infection and colonization of plant species by β-glucuronidase-expressing derivatives of *R. solanacearum*. Plants were grown in non-axenic peat mix (A, B, F, G, K, L, N, O, P, Q and R) or in axenic conditions (C,



D, E, S and T in soft agar; H, I, J, M in liquid solution). Pictures refer to the β-glucuronidase-expressing IPO-1609 strain 1.3 of *R. solanacearum* R3 bv2, unless otherwise mentioned. A and B, Vascular colonization of tomato hypocotyls (cv.

Supermarmande) by derivatives of GMI 1000 (R1 bv 3) and IPO-1609 (R3 bv 2), respectively. C, Infection (black arrow) of a young tuber (white star) at the end of a stolon. D, Invasion of vascular system of a potato stem at 2 mpi. E, Semithin section in the potato stem. Note target bacteria in the xylem vessels (black arrows) and abundant presence of amyloplast (white arrow). F, Infection (black arrow) of a potato tuber bud and vascular invasion (dotted lines) of the tuber stolon meristem in a longitudinal cut (black star) of the tuber. The white star indicates the epidermis of the tuber. G, Transverse cut of a potato stolon showing vascular invasion. H, Xylem colonization in bittersweet nightshade collar. I, Semithin transverse cut of bittersweet nightshade at a root crack of a secondary root with an abundant, intercellular cortical infection (white star) and vascular invasion (black arrow); Lr, longitudinal root. J, Semithin longitudinal section showing intercellular infection of inner cortex at a root tip of bittersweet nightshade (white stars). K, Adsorption on some external tissues in alfalfa. L, Adsorption to the external surface in longitudinal and transverse sections of lower parts of stems in barley. M, Invasion of vascular tissues in kidney bean (white asterisks). N, Vascular colonization of central cylinder in cabbage (white asterisk). O, Semithin section from an infected cabbage stem showing metaxylem colonization (white asterisks). P, Q, R, Adsorption on some external tissues in carrot, fennel and fiber flax, respectively. S, Transverse semithin section of maize root with intercellular infection of the inner root cortex (white star), proximal to an emergent meristem (m) of a lateral root, and the endodermis (e). T, Large infection of cortical tissues in maize by derivative of strain GMI 1000. Target bacteria were visualized in plant tissues either by β-glucuronidase activity in handmade plant sections (A, B, C, F, G, H, K, L, N, P, Q and R), sections cut with a vibratome (D, M and T) or poststaining with methylene and toluidine blue in semithin sections (E, I, J, O and S). Different scale bars are indicated on each picture.

(continued from page 102)

Isolations were negative in all the plants. The taproots collected from the inoculated plants did not differ in size with respect to those from the uninoculated plants.

Fiber flax. For cv. Hermès, under axenic conditions in vermiculite with peat mix, transverse sections at collar level did not show any specific GUS activity in plant tissues, although isolation was positive for one out of eight inoculated individuals (data not shown). From the non-axenic peat mix-grown plants, no blue staining and no bacteria were obtained from the majority of the 82 individuals tested (Fig. 2.1.1 R). Only in four of them, positive isolations were correlated with external contamination following casual splashing of the bacterium onto a rough stem surface (Table 2.1.2).

Field pea. For cv. Solara, from the non-axenic peat mix-grown plants, 11 out of the 36 individuals were positive for the presence of the IPO-1609-Gus on root surfaces and a limited cortex infection was detectable in some root systems without any subsequent xylem invasion. No colonization was observed in the upper levels of the plants, either in the cortex tissues or xylem bundles. Isolations were positive from all the analyzed individuals with an infected cortex (Table 2.1.2).

Horseradish. In the non-axenic peat mix-grown plants, a few root systems from inoculated individuals exhibited an external contamination by the bacterium, but only one of them was invaded in the cortical area, although this infection was not observed in upper levels of the plant. Xylem vessels were never colonized, regardless of the plant level analyzed. Positive isolations in six out of the 36 plants were linked to the presence of the pathogen on the root surface and cortex areas (Table 2.1.2).

Maize. For cv. Symphony, under axenic conditions in soft agar, microscopy of the transverse semi-thin sections of all plant roots showed small, intercellular infection pockets in the cortex, with a slight colonization of the cortical intercellular spaces by bacterial cells (Fig. 2.1.1 S). Transverse sections of the lower part of stems did not show any specific GUS activity in inner plant tissues, although the bacterium was isolated from two out of the 15 inoculated individuals (data not shown). From the non-axenic peat mix-grown plants, a blue staining was only observed on the surface of all maize root systems and lower part of stems, while no *uidA* expression was ever detectable in the transverse sections of the roots and lower plate. Isolations of the pathogen from middle part of the stem were negative for 54 out of the 55 inoculated plants (Table 2.1.2). The positive one was

correlated with presence of the bacterium between the leaves of the sheath. A similar assay carried out with the β-glucuronidase-expressing GMI 1000 strain 9.2 in axenic maize plants grown in soft agar (data not shown) revealed a massive intercellular invasion of cortical tissues, which were particularly highly stained (Fig. 2.1.1 T).

Rutabaga. For cv. Champion, the IPO-1609-Gus was detected in root epidermal and cortical tissues of 24 out of 36 inoculated plants grown in non-axenic peat mix, and in xylem of roots and the lower part of stems in only six of them (Table 2.1.2). For cv. Whilemsburger, most of the inoculated non-axenic peat mix-grown plants showed adsorption on the root surface and infection in the root cortex, but only a few of them colonization of vascular tissues in the roots. Few plants revealed an invaded xylem at the lower part of the stem. Isolations were negative in all the 36 plants analyzed (Table 2.1.2).

Zucchini. For cv. Virginia, from the non-axenic peat mix-grown plants, the labelled bacterium was detected superficially on root systems in a small number of the inoculated individuals, but no infection and invasion of the inner plant tissues occurred, at any plant level. Positive isolations of the IPO-1609-Gus from the stem were attributed to external contamination (Table 2.1.2).

2.1.4 Discussion

We performed histological analyses to describe the localization of *R. solanacearum* R3 bv 2 in various root and/or stem tissues from diverse plants. An accurate interpretation can be ascertained if enough plants are used, which will help to differentiate between true and putative host plants.

Some preliminary assays were carried out under axenic conditions in test tubes with barley, carrot, fennel, fiber flax, kidney bean and maize, in order to compare results with those obtained from the non-axenic peat mix-grown plants. In both types of experiments, bacteria were generally located in the same zones with no significant differences in barley and fiber flax. However, under axenic conditions, kidney bean plants were more frequently and intensely colonized than in non-axenic experiments, and

roots of maize, carrot and fennel were only colonized in axenic and never in non-axenic conditions. Some reasons that may help to explain the differences are: (i) the lack of indigenous microbiota prevents competition and/or predation events, reported to occur in natural soils (Graham *et al.*, 1979; Schonfeld *et al.*, 2003), (ii) soluble plant factors that favor bacterial growth are more easily accessible to the bacterium in liquid MS or in soft agar than in peat mix, (iii) the weaker development of axenic roots in comparison with roots grown in peat mix and (iv) *R. solanacearum* was essentially growing in co-culture with the plants in MS liquid solution or in soft agar. Humidity was extremely high, which strongly favored the bacterium and may have permitted it to survive as an epiphyte or endophyte on plants, even though it could not do so under field conditions. Moreover, axenic liquid culture seems to facilitate infection of intercellular spaces of root cortex, boosting disease development (Vasse *et al.*, 1995). On the whole, these conditions might mimic the status encountered in temporary swampy areas in the lowest part of cultivated fields, where the disease first develops. Consequently, in our experiments using a great many species, we mainly focussed on peat mix-grown plant assays, as these might correlate better with field conditions, in the absence of temporary excess of water.

We used tomato and potato plants as a control for our methodology. In tomato plants cv. Roma, intense invasion of xylem bundles by the IPO-1609-Gus strain 1.3 was visible at the collar level after one week postinoculation and was generalized in all plants by one month. Histological localization of the pathogen was consistent with that described in previous reports (Kelman, 1953; Smith, 1920; Vasse *et al.*, 1995). Our preliminary comparison of root colonization by bv 2 and 3 strains was in agreement with the results by Orozco-Miranda *et al.* (2004), who had already reported that bv 3 strains were better root colonizers than those of bvs 1 and 2 in several plant species.

In potato plants, specific *uidA* staining clearly illustrated that root extremities and lateral root emerging sites were preferably colonized by the pathogen, as previously described in tomato roots, probably due to chemotaxis of *R. solanacearum* to root exudates (Vasse *et al.*, 1995; Yao &

Allen, 2006). Moreover, the endodermis is not yet fully differentiated at these sites and, consequently these root cracks constitute potential points of vascular cylinder infection, as already described in tomato plants (Vasse *et al.*, 1995). In inner plant tissues of potato roots and stems, our results confirmed that the xylem bundles and some parts in the cortex were invaded as illustrated elsewhere (Kelman, 1953; Smith, 1920). Tubers were also infected, exhibiting abundant bacterial ooze in the vascular ring as already reported (Kelman, 1953; Smith, 1920). We clearly demonstrated that eyes and stolon heel ends were the main entrance sites as previously suggested (Kelman, 1953). No differences were observed between cvs. Désirée and Spunta with respect to the histological localization of the pathogen, although bacterial colonization in Spunta took longer to occur.

In bittersweet nightshade plants, blue *uidA* staining was observed in xylem of roots and collars, and also in the root cortex, although in most cases the pathogen was not isolated from the middle part of stem. The presence of the bacterium in the lower parts of the stem but not in upper levels of the plant might be related to the presence of tyloses, microscopically visualized in vascular tissues, which would be limiting bacterial spread as a mechanism of plant defence in response to infection by *R. solanacearum* (Grimault *et al.*, 1994; Kelman, 1953; Smith, 1920). Our results confirm the general statement that bittersweet nightshade is a symptomless carrier of *R. solanacearum* (Elphinstone *et al.*, 1998; Janse, 1996; Kempenaar *et al.*, 1998; Persson, 1998; Stead *et al.*, 1996), although wilting has occasionally been reported (Wenneker *et al.*, 1999).

The other plant species and cultivars were classified in three groups under the evaluated conditions: (i) susceptible hosts, (ii) tolerant hosts and (iii) non-host plants. The first two groups encompass plants which are invaded by high densities of the pathogen in xylem elements at the root level, while invasion of xylem at middle part of stem is heavy in susceptible plants and strongly limited in tolerant plants (Prior *et al.*, 1994; Vasse *et al.*, 2005). The last group refers to non-host plants and includes species in which no xylem invasion was observed, though some external contamination of the rhizoplane and rare cortical infection pockets may occur.

The first group consists of the susceptible hosts. In forage cabbage cv. Proteor, the β-glucuronidase-expressing strain IPO-1609 colonized root cortex and xylem and lower parts of stems and was isolated from middle part of stems in all analyzed plants. In contrast, in cabbage cv. Quintal d'Alsace and curly endive cv. Rida Doble de Verano, *uidA* expression was not observed in root xylem and, with respect to root cortex, it was negative for this cv. of cabbage but positive for curly endive, whereas the specific blue staining was frequently visualized in cortex and xylem from the lower part of stems, and isolations were positive in all plants. Prior to bacterial multiplication *in planta*, low numbers of bacteria in roots and lower parts of stems were probably present but undetected by GUS activity assays. Consequently, all these plants should be considered susceptible to invasion by the tested strain of *R. solanacearum* R3 bv 2 and must be avoided in a crop rotation scheme. It has already been mentioned that curly endive may host *R. solanacearum* bvs 1 and 3, and exhibit symptoms of the disease (Melo & Takatsu, 1997).

The second group consists of the tolerant hosts. In cabbage cv. Virtudes and rutabaga cvs. Champion and Whilemsburger, specific *uidA* staining of inner plant tissues was observed in xylem vessels in roots or in low parts of stems in only a few individuals, and isolations from the middle part of stems were always negative. The related crop cauliflower (*Brassica oleraceae* cv. capitata) has already been recognized as a tolerant host for *R. solanacearum* bvs 1 and 3 (Melo & Takatsu, 1997) and in field trials (Terblanche, 2002). *B. oleraceae* L. has been suggested as a potential biofumigant to reduce *R. solanacearum* bv 3 populations in soil, though with limited success (Arthy *et al.*, 2005). In general, *Brassicaceae* plants have been considered hosts of *R. solanacearum* and are not recommended as a rotation crop (Anonymous, 2000, 2006).

In kidney bean cv. Coco blanc, under axenic conditions an *uidA* expression was visualized both in xylem and cortex in most analyzed plants, while in non-axenic peat mix just one individual was contaminated. Consequently, this cv. should not be considered in crop rotation systems. Likewise, Katafiire *et al.* (2005) reported that beans as a rotation crop were not as effective as cereals in reducing bacterial wilt incidence in the field.

Phaseolus vulgaris L. has been recognized as a host for *R. solanacearum*, but not for bv 2 (Girard *et al.*, 1993). It has been reviewed either as a symptomless carrier for bv 3 of *R. solanacearum* (Hayward, 1994), or as a host for bv 3 (Elphinstone, 2005; Hayward, 1994; Melo & Takatsu, 1997) and also for bv 1 (Melo & Takatsu, 1997) and bv 4 (Hayward, 1994). These conflicting results are probably due to the use of cvs. displaying different susceptibility or of strains from different bvs or with variable levels of aggressiveness.

The third group consists of the non-hosts. In barley cv. Cork, black radish cv. Gros Long d'Hiver, fiber flax cv. Hermès, field bean cv. Maya, field pea cv. Solara, horseradish, maize cv. Symphony and zucchini cv. Virginia the specific *uidA* staining was only observed in some root cortex areas for some of the plant species, but never in inner root or stem tissues. In maize roots, intercellular infection pockets were microscopically visualized for *R. solanacearum* R3 bv 2 and R1 bv 3, although they were more developed by R1 bv 3. The latter results support once again the statement that bv 3 strains can be better root colonizers of some species than bv 2 strains (Orozco-Miranda *et al.*, 2004). Interestingly, both bvs behaved in maize similarly to a non-pathogenic mutant of *R. solanacearum* in tomato (Vasse *et al.*, 1995), indicating that a similar incompatible host-bacterium combination could lead to the formation of restricted cortical infection pockets. Positive isolations of the pathogen were correlated with either root cortex infection or external epidermal presence of the β-glucuronidase-expressing derivative. In barley, intense *uidA* staining was visualized between the leaves, although the bacterium did not enter the plant. Typical sheath structure of *Monocotyledoneae*, with leaves inserted into each other, may constitute a protecting shelter for the bacterium, from where it can be isolated, as it was also found in maize. Likewise, in black radish, fiber flax, field bean, field pea, horseradish and zucchini, a rough surface in the lower part of the stem seems to provide a shelter for the bacterium in highly contaminated plants. All these shelters might favor *R. solanacearum* survival, and should be tested under field conditions to know whether this bacterium can persist in them, acting as reservoirs in the absence of a host. That should be taken into account when selecting

rotation crops, at least in humid areas. One report found that *R. solanacearum* could infect maize roots at a low frequency and that the infection remained localized (Granada & Sequeira, 1983). However, maize and other *Gramineae* have been successfully used to reduce bacterial wilt in a crop rotation system (Katafiire *et al.*, 2005; Verma & Shekhawat, 1991), depending on the level of *R. solanacearum* infestation in soil (Lemaga *et al.*, 2001).

In alfalfa cvs. Gea R1 and Symphonie, carrot cvs. Nantaise and Tempo, celery cv. Istar, colocynth and fennel cv. Doux de Florence, no *uidA* staining by the derivative was observed in inner root or stem plant tissues, although the IPO-1609-Gus was detected on the surface of the roots in these alfalfa, carrot, celery and fennel cvs. Presence of *R. solanacearum* cells on the surface of roots indicates either that root infection was weak and undetected, or points to an actual inability to infect the latter plants. In a crop rotation system, carrots were not as effective as cereals in reducing bacterial wilt incidence (Katafiire *et al.*, 2005), and it has been reported that *R. solanacearum* bvs 1 and 3, but not bv 2 could colonize carrot root surfaces (Melo & Takatsu, 1997). Fennel was reviewed as a host without any reference to the bvs involved (Hayward, 1994). To our knowledge, alfalfa, celery or colocynth, which were non-host plants according to our criteria, had not been previously tested to assess their resistance to *R. solanacearum* R3 bv 2.

In this work, the variability of results within a plant family was remarkable. Plant species belonging to *Brassicaceae* were either susceptible, tolerant or non-host for the β-glucuronidase-expressing derivative of *R. solanacearum* R3 bv 2, while those belonging to *Leguminosae* were either tolerant or non-hosts. Even for a single plant species the results will probably depend on the plant cv. and/or the *R. solanacearum* bv, and within each bv on the aggressiveness of each strain. For this reason it would be advisable to address the host range of the *R. solanacearum* species complex not as a general statement but as a concept which is adapted to a geographical region with its own specific native strains and crops.

The methodology applied in this work, based upon a rapid and simple examination of a great many plant segments embedded into a GUS

reactive agar, proved easy to use. It enabled us to preliminarily classify the plant species as susceptible hosts, tolerant hosts or non-hosts for the bv 2 of the pathogen, on the basis of histological localization *in planta*. On one hand, results pointed out some plant species and cvs. to be susceptible or tolerant for *R. solanacearum* R3 bv 2, which presumably should be avoided as rotation crops. On the other hand, several candidates as non-host plant species and cvs. were suggested, although some of them could be reservoirs of the pathogen under particular conditions. All these candidates should be tested in open-field trials prior to be utilized for rotation schemes aimed at a successful prevention of bacterial wilt damages and spread.

Acknowledgements

B. Álvarez specially thanks the Marie Curie Foundation for a Marie Curie Fellowship at the IFR40 in Toulouse (France) and the Instituto Valenciano de Investigaciones Agrarias for a predoctoral grant. The authors wish to thank S. Poussier for providing with the β-glucuronidase-expressing IPO-1609 strain 1.3, the French Federation for Potato for sending Spunta and Désirée potato axenic seedlings, L. Penya, A. Urbaneja and P. Lemos for technical advice and F. Barraclough for the English language revision.

This work has been funded by the European contract QLK3-CT-2000-01598 acronym "Potatocontrol".

CHAPTER 2.2

Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms

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Microbiology **(2008) 154: 3590–3598**

Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms

Abstract

Survival strategies exhibited over four years by *Ralstonia solanacearum* phylotype (ph) II biovar 2 in environmental water microcosms were examined. The bacterium is a devastating phytopathogen whose ph II biovar 2 causes bacterial wilt in solanaceous crops and ornamental plants. Outbreaks of the disease may originate from pathogen dissemination in watercourses, where it copes with extended nutrient limitation. To ascertain the effect of long-term starvation on survival and pathogenicity of *R. solanacearum* in natural water microcosms, survival experiments were conducted. Microcosms were prepared from different sterile river water samples, and inoculated separately with two European strains of ph II at 10^6 c.f.u. ml^{-1} at 24ºC over four years. In all assayed waters, starved *R. solanacearum* kept in a non-growing but culturable state within the first year, maintaining approximately their initial numbers. Afterwards, part of *R. solanacearum* culturable population progressively lost the ability to form colonies and non-culturable but metabolically active cells were appearing. During the whole period, the bacterium remained pathogenic on host plants and underwent a transition from typical bacilli to small cocci which tended to aggregate. Some starved *R. solanacearum* cells filamentated and formed buds. Starvation response, viable but non-culturable state, morphological changes and aggregation were survival mechanisms firstly reported for this pathogen as induced in oligotrophic conditions. The potential existence of long-starved pathogenic cells in environmental waters may raise new concerns on the epidemiology of the bacterial wilt disease.

2.2.1 Introduction

Bacterial wilt affects numerous crops and some ornamental plants worldwide (Elphinstone, 2005; Hayward, 1994). The causative agent is the *Ralstonia solanacearum* species complex (Fegan & Prior, 2005), classified into phylotypes and biovars according to molecular and biochemical characteristics respectively (Fegan & Prior, 2005; Hayward, 1991). It is considered a quarantine organism in the European Union (Anonymous, 2000) and a bioterrorism agent in the U.S.A. (Lambert, 2002). The bacterium is soil and water borne, waterways being major dissemination routes in the environment (Elphinstone, 2005). It enters the plant through the roots and colonizes the vascular system producing severe disease (Hayward, 1991; Kelman, 1953). After destroying the plant, an inter-host life period has been proposed for this pathogen (Denny *et al.*, 1994), where it would have to cope with the severity of oligotrophic habitats. Survival studies in sterile water report persistence of the bacterium for variable periods (Kelman, 1956; van Elsas *et al.*, 2001; van Overbeek *et al.*, 2004; Wakimoto *et al.*, 1982), and occasional capacity to wilt susceptible hosts (Caruso *et al.*, 2005; van Overbeek *et al.*, 2004). Although some environmental factors affecting *R. solanacearum* physiology have been reviewed (Hayward, 1991; Kelman, 1953; van Elsas *et al.*, 2005), knowledge on the survival strategies exhibited by this pathogen under prevailing starvation in aquatic habitats is still scarce (López & Biosca, 2005).

Microorganisms can resort to diverse mechanisms when confronted with nutrient deprivation (Morita, 1997). Several starvation-survival responses have been described, with starved bacterial populations maintaining their numbers over time in a non-growing but culturable state at different levels depending on the species (Morita, 1997). This starvation-induced state for survival proved to be distinctly different from that of active growth (Heim *et al.*, 2002). Under prolonged oligotrophy, reduction in cell size and change to a round cell shape are also considered as bacterial strategies to survive in the environment (Novitsky & Morita, 1976; Rollins & Colwell, 1986; Ruiz *et al.*, 2001), as well as the induction of a viable but non-culturable (VBNC) state (Oliver, 2005; Roszak & Colwell, 1987) that

constitutes a distinct physiological state from that of the starvation response (Heim *et al.*, 2002). *R. solanacearum* becomes VBNC by exposure to copper (Grey & Steck, 2001) and after incubation at low temperature in soil and water (Caruso *et al.*, 2005; van Elsas *et al.*, 2000, 2001; van Overbeek *et al.*, 2004). Adaptations to oligotrophy have not yet been described for *R. solanacearum*.

The occurrence of disease outbreaks linked to the presence of *R. solanacearum* phylotype (ph) II biovar (bv) 2 in European waterways (Caruso *et al.*, 2005; van Elsas *et al.*, 2000, 2001), together with the existence of European Union Directives banning irrigation of host plants with contaminated water (Anonymous, 1998, 2000, 2006) make the establishment of *R. solanacearum* in non-host environments a matter of concern. As the life of *R. solanacearum* in water remains still rather unknown, this work aimed to investigate the survival mechanisms exhibited by the ph II bv 2 of this pathogen in response to nutrient deprivation in environmental water microcosms over four years.

2.2.2 Materials & Methods

2.2.2.1 Bacterial strains and culture conditions

Strain IVIA-1602.1 of *R. solanacearum* ph II bv 2 isolated from potatoes with brown rot symptoms in Spain was used in all experiments, and the Dutch strain IPO-1609 (also ph II bv 2) (van Elsas *et al.*, 2001) kindly provided by J. D. van Elsas, was included for comparison. Strains were kept at –80 ºC in a 30% (v/v) glycerol medium and were grown on non-selective Yeast Peptone Glucose Agar (YPGA) (Ridé, 1969) with filtered-sterilized glucose, for 72 h at 29ºC.

2.2.2.2 Characteristics of water samples

Four river water samples were collected from different sites in Spain (Caruso *et al.*, 2005), and each of them was separately used for survival experiments. Mineral nutrient concentrations ranged as follows, depending on the water sample (values per litre): Na, 5.7-12.2 mg; K, 1.4-1.6 mg; Mg,

3.5-3.8 mg; Ca, 10.3-12.2 mg; Fe, 0.21-0.25 mg; Mn, 0.04-0.06 mg; nitrates, 4.5-6.8 mg, and phosphates, 0.24-0.36 mg. Organic matter contents estimated by biochemical oxygen demand were <1.8 mg O_2 l^{-1}. In the different water samples pH values were around neutrality, from 6.5 to 7.3.

2.2.2.3 Preparation of water microcosms

Survival experiments were conducted similarly to Caruso *et al.* (2005). For each of the four water samples three microcosms were prepared. Every single microcosm was composed of 200 ml of river water filtered through 0.22 μm pore size, autoclaved, and then inoculated with the strain IVIA-1602.1 or IPO-1609 in phosphate buffered saline (PBS) at pH 7.2, to reach a final concentration of 0.5% ($5x10^6$ c.f.u. ml^{-1}). After inoculation, the microcosms were incubated for 4 years at 24±1ºC. This temperature was selected because it was the nearest to the *R. solanacearum* optimal that had been registered in some Spanish rivers in warm months (Caruso *et al.*, 2005).

2.2.2.4 Bacterial cell counts

Microscopic counts for total and viable *R. solanacearum* cells and plate counts for culturability were performed from each microcosm as described (Álvarez *et al.*, 2007) at inoculation time (day 0) and days 1, 2, 4, 8, 14, 28 and 40, also at 2, 3 and 6 months, and every 6 months up to 4 years of incubation. For total and viable cell counts, aliquots from the microcoms were incubated with yeast extract and nalidixic acid according to a Direct Viable Count (DVC) method (Kogure *et al.*, 1979), for 16 h (van Elsas *et al.*, 2001) except with long-starved cells, for which the incubation was prolonged up to 72 h. Cells were fixed by formaldehyde 2% (v/v) and stained with acridine orange 0.1% (w/v) (Oliver, 1987). Long-starved cells were additionally stained by indirect immunofluorescence (Anonymous, 1998, 2006), with a polyclonal antiserum 1546-H IVIA against *R. solanacearum* (Caruso *et al.*, 2005), to determine possible changes in surface antigens. Viable cells were also measured by the Live/Dead® BacLight™ Bacterial Viability Kit (Molecular Probes, Leiden, The Netherlands) in some of the assays. After mixing cells and dyes (SYTO 9 and propidium iodide)

bacterial cells can be counted as alive or dead in terms of membrane integrity (Boulos *et al.*, 1999). Mixtures were incubated in the dark at 24±1ºC for 15 min, and up to one hour in the case of long-starved cells. Bacterial cells were visualized with a Leika epifluorescence microscope at an amplification of x1250 and counted in at least 20 random fields. For culturability, plate counts on YPGA were done after at least 72 h at 29 ºC.

2.2.2.5 Cell size and shape

Microcosm aliquots were taken at some sampling times and cells were directly fixed and stained either by immunofluorescence or acridine orange as abovementioned, without being incubated for viability. *R. solanacearum* cell morphology was observed with a Nikon Eclipse E800 microscope at a magnification of x1000. Pictures were taken with an adapted digital camera using ACT-1 for DXM1200 software, and no processing of the images was performed. Cell size was determined according to the software Image-Pro® Plus (Media Cybernetics, Inc.). For each sample, at least 300 cells were measured in length and width, and cell volumes were calculated.

2.2.2.6 *In planta* pathogenicity assays

(i) Stem-inoculation. Microcosm aliquots were stem-inoculated in non-axenic tomato plants susceptible to *R. solanacearum* (*Lycopersicon esculentum* cv. Roma) at initial time, one, two, three and six months, one year, and then every six months up to four years of microcosm incubation. Inoculations were performed by injecting into the stem 10 µl directly taken from the water microcosms during the first year, and 50 µl from then and until the end of the fourth year. Groups of 14 plants grown in greenhouse conditions from *R. solanacearum*-tested free seeds were used. All stem-inoculations were done with aliquots from strain IVIA-1602.1-inoculated microcosms from the four water samples, in triplicate. For comparative purposes, assays with aliquots from strain IPO-1609-inoculated microcosms were also performed. In all cases, PBS and a bacterial suspension of either strain IVIA-1602.1 or IPO-1609 as negative and positive controls were used. During incubation in a growth chamber (26ºC, 16 h, day; 22ºC, 8 h, night;

70% RH) under quarantine conditions, inoculated plants were periodically monitored up to six weeks for appearance of symptoms and disease development. Stems from wilted plants were cut at different levels above the inoculation point and segments of 2-3 cm were analyzed for presence of *R. solanacearum*. Extracts were plated onto a modified semiselective medium South Africa (SMSA) agar (Elphinstone *et al.*, 1996) and colonies were PCR-tested (OLI1 Y2 primers) as described (Anonymous, 1998, 2006). Stems from inoculated non-wilted plants were processed in the same way after six weeks.

(ii) Watering inoculation. Volumes of 20 ml from the microcosms were used after one-fold dilution, to water groups of 10 tomato plants cv. Roma at time zero and after two and four years of microcosm incubation. Assays were performed with water microcosms inoculated with either the strain IVIA-1602.1 or IPO-1609. Controls, monitoring and analyses of plants were as abovementioned.

2.2.2.7 Statistical analysis

Each survival and pathogenicity assay was done independently with *R. solanacearum*-inoculated microcosms from four different water samples in triplicate. For survival experiments, log-transformed data of total, viable and culturable *R. solanacearum* cell counts were analyzed by using mean values. Significant differences were assessed by variance analysis (ANOVA). Factors considered for the analysis were: water sample, time of incubation and bacterial strain. For pathogenicity experiments, results were analyzed by the Fisher's exact test for categorical data to study possible differences between the two inoculation methods, the two bacterial strains and among the four water samples. In all cases, differences were recorded as significant at $P < 0.05$.

2.2.3 Results

2.2.3.1 Effect of long-term starvation on *R. solanacearum* survival in environmental water microcosms

Within one, two, three and six months, and approximately by the first year post-inoculation in river water microcosms at 24ºC, total *R. solanacearum* strain IVIA-1602.1 populations remained approximately at 2-6x10^7 cells ml^{-1} (Fig. 2.2.1). In this period, metabolically active cells measured by DVC method kept around 1-2x10^7 cells ml^{-1} (Fig. 2.2.1), with comparable values by Live/Dead kit, and culturability ranged between 3-8x10^6 c.f.u. ml^{-1}, depending on the water sample (Fig. 2.2.1). Within the second, third and fourth year, the numbers of total bacterial cells slightly increased up to values from 9x10^7 to 2x10^8 cells ml^{-1} (Fig. 2.2.1). In this period and until 30-36 months post-inoculation, viable *R. solanacearum* cells measured by DVC method and Live/Dead kit also increased, keeping levels around 0.5-1 log units under the total cell numbers. From 30-36 to 48 months, decreases in nutrient responsive cells to values around 1-3x10^6 cells ml^{-1} were observed (Fig. 2.2.1), while Live/Dead counts revealed roughly 3-6x10^7 cells ml^{-1} keeping intact membranes, depending on the water sample. Culturable *R. solanacearum* populations declined approximately from the first year post-inoculation to the end of the experiments, until values from 8x10^3 to 4x10^4 c.f.u. ml^{-1} (Fig. 2.2.1). For each water sample, significant differences ($P < 0.05$) were observed among total, viable and culturable counts although trends in total, viable and culturable populations among the four water samples kept similar ($P > 0.05$) (Fig. 2.2.1). The stronger decreases in culturability with respect to viability ($P < 0.05$) in all waters assayed pointed out the entrance of portions of the bacterial populations into the VBNC state. On plates, the colonies of *R. solanacearum* that were still culturable had a typical smooth morphology with a normal size.

In the comparative experiments using the IPO-1609 strain, survival curves revealed trends similar to those of the strain IVIA-1602.1 ($P > 0.05$) (data not shown).

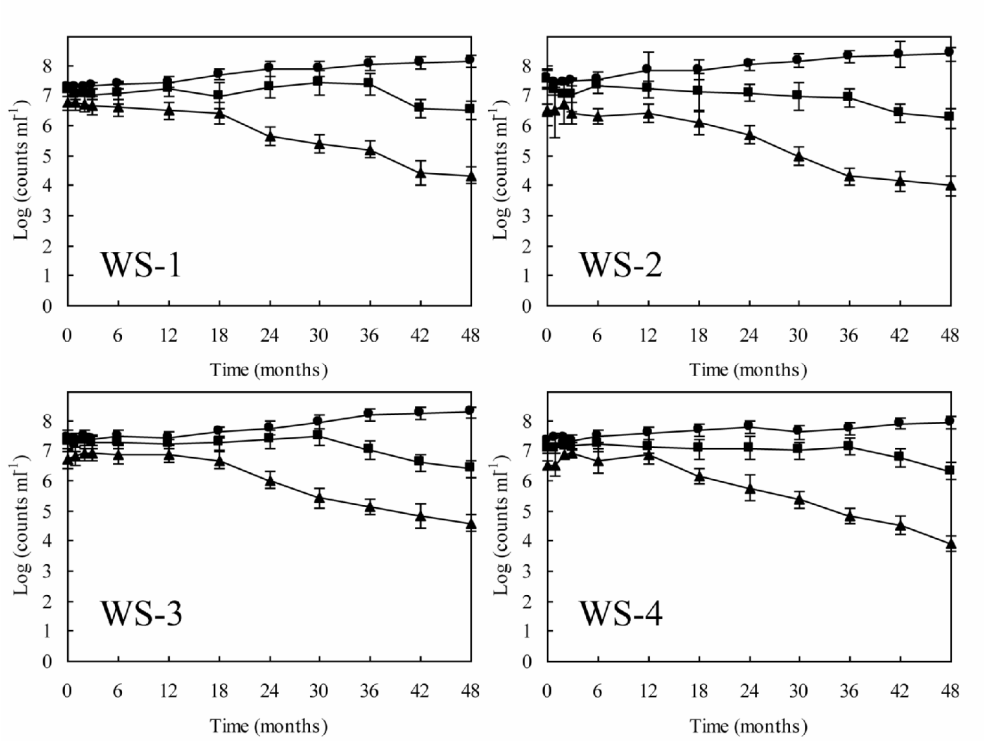

Figure 2.2.1. Survival curves of *R. solanacearum* ph II bv 2 strain IVIA-1602.1 over four years in river water microcosms at 24°C: WS-1, water sample 1; WS-2, water sample 2; WS-3, water sample 3; and WS-4, water sample 4. Total (●) and viable (■) cells, and colony forming units on YPGA (▲). Represented points are means ± standard deviation of triplicate microcosms.

2.2.3.2 Effect of long-term starvation on *R. solanacearum* cell morphology in environmental water microcosms

Freshly growing cells of *R. solanacearum* strain IVIA-1602.1 have a bacillar form (Fig. 2.2.2 A). However, since the first days of starvation and depending on the water sample, coccoid forms were observed up to 1.7 %, progressively increasing to 7-10 % by the first week and to 25-30 % by one

month (Fig. 2.2.2 B). By one year coccoid forms had increased to about 40-50%, to 60-70 % by the second year, and to 80-90 % after four years (Fig. 2.2.2 C and D), with a general tendency to aggregate (Fig. 2.2.2 E). At inoculation time cells had the following mean size values: length, 1.5 ± 0.3 μm (± std. dev.); width, 0.5 ± 0.1 μm; and volume, 0.20 ± 0.07 μm^3. After four years cells had reduced their size, showing a mean diameter of 0.65 ± 0.2 μm and a mean volume of 0.14 ± 0.08 μm^3. Volume reduction ranged from 18 to 77 %.

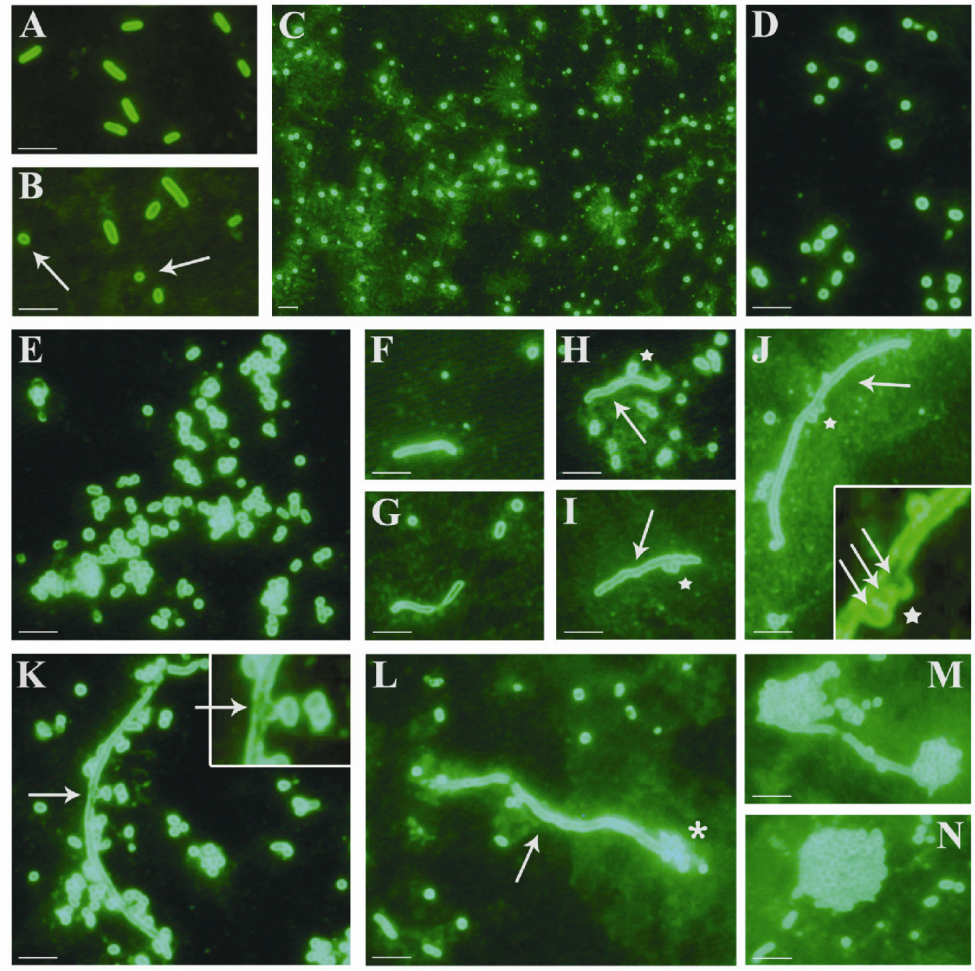

Figure 2.2.2. Microscopic observations of the changes in morphology of *R. solanacearum* ph II bv 2 cells of strain IVIA-1602.1 under starvation in river water microcosms at 24ºC. (A) Freshly grown cells, bacillar typical form. (B) One-month starved cells, co-existence of bacillar and coccoid forms (arrows). (C) Four-year

starved cells, majority of coccoid forms. (D) Detail of coccoid forms by the fourth year. (E) Aggregates of coccoid forms. (F) Filamentous cells formed individually or (G) in pairs. (H, I, J) Suspensor cells (arrows) with buds (stars), either one (H), two (I) or more (J) per cell. (K) Suspensor cell developing a bud (arrow) surrounded by coccoid forms and small aggregates. (J and K, magnifications) Basal parts of developing buds lacking the cell wall (arrows). (L) Coccoids at the extremities of the suspensor cell (arrow), either as incipient aggregates (asterisk) or (M) developing aggregates. (N) Detail of a well-formed aggregate. Bacteria were visualized at a magnification of x1000 after specific immunofluorencence staining. Scale bars: 2.5 μm.

During the long-term starvation, filamentated, elongated forms of *R. solanacearum* were observed (Fig. 2.2.2 F and G). Some longer filamentous cells showed localized expansions or buds which maintained cell wall, as evidenced by immunofluorescence observation (Fig. 2.2.2 H, I and J). This type of cells has been referred to as suspensor cells (Thomas *et al.*, 1999). Whereas individual filamentous cells were observed to form buds along the cell (Fig. 2.2.2 H, I and J), sometimes surrounded by coccoid aggregates (Fig. 2.2.2 K), filamentous cells in pairs had the budding points at their extremities (Fig. 2.2.2 L and M). Well-formed aggregates were, at least superficially, composed of coccoid forms (Fig. 2.2.2 N). Acridine orange staining showed that filamentous cells, coccoids, buds and aggregates contained nucleic acids. In comparison, strain IPO-1609 similarly changed shape, reduced size and filamentated.

2.2.3.3 Effect of long-term starvation in environmental water microcosms on *R. solanacearum* pathogenicity and water-borne transmission

Cells of *R. solanacearum* strain IVIA-1602.1 starved in microcosms from different water samples kept disease-inducing capacity for up to the fourth year, as shown in Table 2.2.1. As expected, wilting percentages were significantly ($P < 0.05$) higher by stem-inoculation than by watering, except at time zero (Table 2.2.1). For stem-inoculation, in general and regardless of

the time period that cells were under starvation, tomato plants started wilting at around one week, with a similar pace of symptom development with respect to the positive controls. At approximately two weeks post-inoculation, about 25-40 % of the inoculated tomato plants were wilting and by three weeks 80-100 % showed severe disease (Table 2.2.1); the pathogen was reisolated from all of them on SMSA, and PCR-identified. On plates, colonies of *R. solanacearum* were typically smooth. The inoculated tomato plants remaining nonwilted after six weeks (up to 20 %) were also processed, and the bacterium was detected in about 70 % of them at distances from two to 16 cm of the inoculation point, proving the infectivity and spread of the inoculum. Differences observed among the four water samples were not significant ($P > 0.05$) (Table 2.2.1).

In watering inoculation assays, *R. solanacearum* strain IVIA-1602.1 starved cells caused wilting of the tomato plants within six weeks after plant inoculation (Table 2.2.1), even four-year starved cells (17-47 %). Appearance of symptoms was roughly at two weeks post-inoculation, with an irregular pace of disease spread in the sets of plants in the different water samples (data not shown). The pathogen was reisolated and identified from the wilted plants, and colonies grown on SMSA were smooth.

Table 2.2.1. *In planta* pathogenicity assays of *R. solanacearum* ph II bv 2 strain IVIA-1602.1 long-starved in river water microcosms, on tomato plants cv. Roma. Water microcosms were from: WS-1, water sample 1; WS-2, water sample 2; WS-3, water sample 3; and WS-4, water sample 4. Control plants inoculated with *R. solanacearum* strain IVIA-1602.1 yielded 100% wilting in stem-inoculation and 93-100% in watering inoculation. Plants inoculated with PBS were negative. Data represent mean values from triplicate assays. Standard deviation intervals: for *R. solanacearum* plant inocula were ± 0.16 log (c.f.u./plant) and ± 0.05 log (c.f.u./pot); and for wilting were ± 3.34 % in stem-inoculation and ± 5.95 % in watering inoculation. Value corresponding to 100% (stem inoculation) = 42 tomato plants (14x3 sets of plants). Value corresponding to 100% (watering inoculation) = 30 tomato plants (10x3 sets of plants).

Starvation period (months)	*R. solanacearum*-inoculated river water microcosms from:							
	WS-1		WS-2		WS-3		WS-4	
	STEM INOCULATION							
	Inoculum (cfu/plant)	Wilting (%)	Inoculum (cfu/plant)	Wilting (%)	Inoculum (cfu/plant)	Wilting (%)	Inoculum (cfu/plant)	Wilting (%)
0	5.50x10e4	100	2.09x10e4	100	5.13x10e4	100	3.24x10e4	100
1	5.62x10e4	100	2.00x10e4	100	6.76x10e4	100	3.39x10e4	100
2	5.37x10e4	98	3.16x10e4	100	8.13x10e4	100	7.76x10e4	100
3	4.47x10e4	95	1.59x10e4	98	8.51x10e4	98	7.94x10e4	98
6	3.98x10e4	98	1.26x10e4	100	7.59x10e4	98	4.47x10e4	98
12	3.16x10e4	95	1.59x10e4	100	7.76x10e4	98	6.92x10e4	93
18	1.26x10e5	100	5.00x10e4	91	2.34x10e5	100	6.90x10e4	88
24	2.19x10e4	95	2.51x10e4	83	5.25x10e4	93	2.95x10e4	91
30	1.20x10e4	93	5.00x10e3	88	1.35x10e4	86	1.20x10e4	86
36	8.10x10e3	86	1.00x10e3	93	4.16x10e3	95	3.31x10e3	81
42	1.32x10e3	88	7.05x10e2	93	2.40x10e3	88	1.62x10e3	83
48	1.10x10e3	83	5.00x10e2	95	2.00x10e3	91	4.00x10e2	81
	WATERING INOCULATION							
	Inoculum (cfu/pot)	Wilting (%)	Inoculum (cfu/pot)	Wilting (%)	Inoculum (cfu/pot)	Wilting (%)	Inoculum (cfu/pot)	Wilting (%)
0	1.10x10e7	93	4.18x10e6	87	1.03x10e7	93	6.48x10e6	90
24	8.74x10e5	73	1.00x10e6	70	2.10x10e6	53	1.18x10e6	67
48	4.38x10e4	47	2.00x10e4	40	8.00x10e4	20	1.60x10e4	17

(continued from page 128)

In stem and watering inoculations, negative control plants did not show any disease symptom.

Comparative pathogenicity assays performed with the Dutch IPO-1609 strain yielded similar results in stem-inoculation ($P > 0.05$) (76-91% of the tomato plants by the fourth year, depending on the water sample). It was also capable of water-borne transmission of the disease, although in a lesser extent (10-23 % of the plants by the fourth year, depending on the water sample).

2.2.4 Discussion

R. solanacearum ph II bv 2 survival strategies under long-term exposure to oligotrophic conditions were firstly investigated in environmental water microcosms. To our knowledge, this is the longest monitoring of *R. solanacearum* behaviour in a non-host environment, supporting the great potential for persistence of this pathogen keeping disease-inducing capacity.

R. solanacearum was able to survive under conditions of extended nutrient limitation in sterile river waters exhibiting diverse survival mechanisms. One of them was a starvation response consisting of maintaining populations in a non-growing but culturable state, as commonly described for other bacterial species (Morita, 1997) but not for *R. solanacearum* as such up until now. Previous studies on survival of the pathogen were performed in sterile pure water (Kelman, 1956; van Overbeek *et al.*, 2004; Wakimoto *et al.*, 1982) or sterile natural water only for several months (Kelman, 1956; van Elsas *et al.*, 2001) to a maximum of one year (Tanaka & Noda, 1973). Notwithstanding, during the starvation period proportions of the viable *R. solanacearum* populations progressively lost the ability to form colonies on plates, as already observed in starved bacteria (Besnard *et al.*, 2000; Colwell & Huq, 1994), becoming VBNC as another survival strategy under prolonged oligotrophy. Nutrient deprivation has been recognized as an important stimulus to induce the VBNC state, which represents a common strategy for bacterial survival in energy-deficient environments (Colwell & Huq, 1994; Oliver, 2005).

Starvation as VBNC-inducing factor had not been reported so far for *R. solanacearum*.

Total and viable *R. solanacearum* population increase during long starvation would be related to the occurrence of cell divisions, observed for other bacterial species under nutrient deprivation (Byrd, 2000) and considered a survival strategy for bacteria facing adverse environmental conditions (Byrd, 2000; Morita, 1997; Roszak & Colwell, 1987). Multiplying the number of individuals raises the probability of one surviving cell, enabling dissemination and colonization of new ecological niches, which implies a selective advantage for diversification of a bacterial species (Morita, 1997). These cell divisions might have been produced by cryptic growth (Roszak & Colwell, 1987) at the expense of cell debris resulting from the degradation of non-viable cells in the water microcosms. For most of the four years a great many starved *R. solanacearum* cells kept membrane permeability and proved to be nutrient-responsive, being capable of protein and nucleic acid synthesis as stated for other bacteria (Kogure *et al.*, 1979). Only towards the end of the period, the appearance of starved cells keeping intact membranes (according to Live/Dead staining) but being unable to metabolize the added substrate (according to DVC determination) might indicate that metabolic response is likely to be a more stringent viability criterion than membrane integrity, as suggested (Boulos *et al.*, 1999).

During long starvation, *R. solanacearum* bacilli underwent a progressive transition to coccoids as in other bacteria (Chaiyanan *et al.*, 2007; Novitsky & Morita, 1976; Rollins & Colwell, 1986; Ruiz *et al.*, 2001). Cell volume progressively decreased without significant antigenic change, since cells were readily immunostained, similarly to two-year starved *Vibrio cholerae* (Chaiyanan *et al.*, 2007). Cell round shape and size reduction are assumed to be survival responses as a result of cell divisions, to minimize cell maintenance and better capture scarce nutrients (Chaiyanan *et al.*, 2007; Morita, 1997; Roszak & Colwell, 1987). In *R. solanacearum*, size reduction and rise in total and viable cell populations would have been caused by reductive divisions, as commonly reported under long starvation (Byrd, 2000; Morita, 1997; Roszak & Colwell, 1987). That represents a

survival advantage, since a higher number of small and round cells may penetrate narrower and deeper sites looking for food and protecting themselves from predators (Byrd, 2000; Morita, 1997) favouring species perpetuation. The increase in numbers might have been also produced by filamentation and budding phenomena. *R. solanacearum* filamentated under starvation conditions, as reported for *Escherichia coli* (Wainwright *et al.*, 1999) and *Pseudomonas oleovorans* (Ruiz *et al.*, 2001). Some of the *R. solanacearum* filamentous cells were observed to have cell wall evaginations or buds, similarly to starved *Campylobacter jejuni* (Lázaro *et al.*, 1999; Thomas *et al.*, 1999) and *V. cholerae* (Chaiyanan *et al.*, 2007); these budded cells are considered as transients in coccoid formation (Thomas *et al.*, 1999). *R. solanacearum* filamentous cells were visualized by immunostaining equally to bacillar and coccoid cells, as suggested (Thomas *et al.*, 1999). This is the first report on filamentation and budding phenomena occurring in a plant pathogenic bacterium.

Many of the coccoid forms were viable, although non-viable were also observed. In our belief and in agreement with Shleeva *et al.* (2002), in *R. solanacearum* VBNC populations there would be a co-existence of cells viable enough to be resuscitated and of cells just dying-off. As other bacterial species (Colwell & Huq, 1994; Chaiyanan *et al.*, 2007; Morita, 1997), *R. solanacearum* coccoids showed a tendency to aggregate under starvation, which can be considered a survival mechanism, since cell aggregation enables to utilize the content of lysing cells and protects from environmental hazards such as free radicals, toxins, and predators (Blat & Eisenbach, 1995).

R. solanacearum retained virulence during the four years subjected to nutrient scarcity in river water microcosms. It had previously been reported to be able to wilt host plants after 132-day incubation in ultrapure water (van Overbeek *et al.*, 2004). The similar disease incidence on stem-inoculated plants with *R. solanacearum* either starved (from microcosms) or freshly grown (from controls) during the four years demonstrated the great capacity of this pathogen to cause disease. Interestingly, bacterial wilt was also observed in host plants after watering with long-starved *R.*

solanacearum, which constitutes a more natural way of transmission of this pathogen. Therefore, a hypothetical water-borne dissemination of disease-causing *R. solanacearum* after extended periods of starvation in environmental waters should not be ruled out, as suggested by field studies (Caruso *et al.*, 2005; Hong *et al.*, 2005).

Oligotrophy is the fundamental factor limiting bacterial survival in open aquatic systems (Morita, 1997), but there are other environmental factors playing a role. The influence of temperature, incident light, presence of sediment, seawater salts and aquatic microbiota on *R. solanacearum* populations was monitored, also in water microcosms (Álvarez *et al.*, 2007; van Elsas *et al.*, 2001; van Overbeek *et al.*, 2004). Among them, only temperature has been assessed in natural watersystems, causing a seasonal variation of *R. solanacearum* populations in temperate areas (Caruso *et al.*, 2005; Hong *et al.*, 2005) which did not prevent the survival of the pathogen, even in the presence of aquatic microorganisms.

Although this work was performed under natural oligotrophic conditions in a confined system, the great ability observed for *R. solanacearum* to persist and cause disease is in agreement with studies in open natural waters where the bacterium was detected for several consecutive years (Caruso *et al.*, 2005; Hong *et al.*, 2005). To date, the strategies used by starved *R. solanacearum* ph II bv 2 to survive until contact with the next host were quite unkown. This work unveiled a high capacity of this pathogen to adapt itself to prolonged nutrient scarcity in natural water microcosms. It displayed an efficient starvation response, maintaining a non-growing but culturable population over four years retaining pathogenicity, but also going into a viable but non-culturable state. It increased the number of individuals to favour dispersion, reduced its size to reach smaller places, changed from bacilli to cocci to better capture scarce nutrients and aggregated for protection. Overall, this work provides a broader understanding on the long-term survival of *R. solanacearum* under the oligotrophic conditions characteristic of natural waters, which eventually may improve measures to prevent the dissemination of this devastating pathogen in watersystems.

Acknowledgements

B. Álvarez thanks the Instituto Valenciano de Investigaciones Agrarias for a predoctoral grant. The authors wish to thank J.L. Palomo and the Consejería de Agricultura de Castilla-León for collecting the river water samples, J.D. van Elsas for sending strain IPO-1609 and E. Carbonell, J. Pérez Panadés and J.L. Díez for statistical analysis.

This work has been funded by projects FAIR 5-CT97-3632 and QLK 3-CT-2000-01598 of the European Union, FD 1997-2279 of the Ministerio de Educación y Ciencia of Spain and GV05/214 of the Generalitat Valenciana.

CHAPTER 2.3

Adaptive responses by *Ralstonia solanacearum* phylotype II confronted with environmental temperatures in freshwater microcosms

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To be submitted

Adaptive responses by *Ralstonia solanacearum* phylotype II confronted with environmental temperatures in freshwater microcosms

Abstract

Ralstonia solanacearum phylotype II adaptations under exposure to different temperatures in nutrient-limiting environmental waters were examined. The bacterium is a devastating phytopathogen with an economically important host range wordwide. Survival experiments were performed in sterile river water and distilled water microcosms separately inoculated with two European strains isolated from either cold or warm habitats, and incubated at low (4ºC), temperate (14ºC) and warm (24ºC) temperatures for 40 days. Adaptation at 4ºC was that of a viable-but-non-culturable (VBNC) induction, dependent on water nutrient contents since the loss of culturability was significantly more slowly in environmental waters, and occurring without relevant changes in typical bacillar morphology. Adaptations at 14ºC and 24ºC consisted of starvation-survival responses, with cells readily culturable in their initial numbers and proportions of coccoids significantly higher at 24ºC. On the host, starved cells kept virulent at 4ºC, 14ºC and 24ºC. The strains behaved similarly regardless of their cold or warm areas of isolation. This work firstly describes natural nutrient availability of environmental waters favouring survival of cold-stressed *R. solanacearum* phylotype II, and distinct temperature influence in the starvation-survival responses. These adaptations of the pathogen to environmental temperatures and oligotrophy did not interfere with its disease-inducing capacity.

2.3.1 Introduction

Bacterial wilt caused by the *Ralstonia solanacearum* species complex (Fegan & Prior, 2005) is one of the world's most serious plant diseases (Kelman, 1953; Hayward, 1991; Brown, 2009) concerning food crops (Elphinstone, 2005) and ornamentals (Swanson *et al.*, 2005). It has a quarantine status in the European Union (EU), the USA and Canada (Anonymous, 2000; Swanson *et al.*, 2005; Brown, 2009), and is considered a priority pathogen for control and containment by the USDA (2007). The complex comprises four phylotypes and five biovars according to molecular and biochemical characteristics repectively (Hayward, 1991; Fegan & Prior, 2005). In temperate environments, *R. solanacearum* phylotype (ph) II biovar (bv) 2 appears to persist during variable periods as a free-living form or associated to non-host roots (Hayward, 1991; Wenneker *et al.*, 1999; Caruso *et al.*, 2005; Elphinstone, 2005; Hong *et al.*, 2005) despite unfavorable conditions compromising the endurance of the bacterium, such as sustained oligotrophy and temperatures below the optimum. Notwithstanding, it was consistently detected from surface run-off waters long after its introduction, keeping pathogenic (Elphinstone *et al.*, 1998; Caruso *et al.*, 2005; Hong *et al.*, 2005).

Reduced nutrient availability characteristic of environmental waters has long been claimed to affect bacterial survival in natural settings (Roszak & Colwell, 1987; Morita, 1997). Although *R. solanacearum* can persist in water for different periods (Kelman, 1956; Tanaka & Noda, 1973; Wakimoto *et al.*, 1982; van Elsas *et al.*, 2001; van Overbeek *et al.*, 2004; Álvarez *et al.*, 2007), only recently it was shown to be able to survive and remain pathogenic in natural waters up to four years displaying strategies such starvation-survival responses, induction of a viable-but-non-culturable (VBNC) state, transition to coccoid cells and aggregation (Álvarez *et al.*, 2008), mechanisms evolved by non-sporulating bacteria facing adverse environmental conditions (Roszak & Colwell, 1987; Morita, 1997; Oliver, 2005; Chaiyanan *et al.*, 2007).

Knowledge on effects of temperatures microorganisms are submitted to in natural settings is crucial to understand temperature

adaptation and survival of bacteria. In environmental watercourses, *R. solanacearum* population levels seasonally varied according to a range of temperatures (Caruso *et al.*, 2005; Hong *et al.*, 2005), with successful isolation at water temperatures above 14ºC but unsuccessful below (Wenneker *et al.*, 1999; Caruso *et al.*, 2005). In agricultural water microcosms, persistence of the pathogen confronted with different temperatures was variable (van Elsas *et al.*, 2001). However, the effect of low temperatures commonly occurring in environmental waters on survival of the pathogen remains poorly understood. Although exposure of *R. solanacearum* to 4ºC in pure water caused the pathogen to become VBNC (van Elsas *et al.*, 2001; van Overbeek *et al.*, 2004), up to date the dynamics of this process has not been monitored in more realistic environmental water microcosms.

Since understanding how environmental prevailing factors affect the fitness of *R. solanacearum* in aquatic habitats is of considerable interest for preventing water-borne dissemination of this deadly pathogen (Brown, 2009), this work addressed the survival ability of two European *R. solanacearum* ph II bv 2 strains from either cold or warm habitats in cold, temperate and warm environmental waters under natural nutrient-limiting conditions, which was compared to that in non-environmental water.

2.3.2 Materials & Methods

2.3.2.1 Bacterial strains and culture conditions

In all survival experiments strains IVIA-1602.1 and IPO-1609 of *R. solanacearum* ph II bv 2, isolated from diseased potatoes respectively in warm (Canary Islands, Spain) and cold (The Netherlands) habitats, were used. The strains have different REP and BOX profiles, and distinct PFGE patterns (Caruso, 2005). They were kept at -80ºC, and then grown on the non-selective Yeast Peptone Glucose Agar (YPGA) (Ridé, 1969) for 72 h at 29ºC.

2.3.2.2 Characteristics of water samples

Four river water samples were collected according to Caruso *et al.* (2005) from different locations in Spain, and nutrient contents were separately determined for each of them. In the different water samples, organic matter levels were from 2 to 3.73 % (w/v), and the main ion concentrations ranged as follows (values per litre): Na^+, 9.7-9.9 mg; K^+, 2.1-2.9 mg; Ca^{2+}, 10.1-13.0 mg; Mg^{2+}, 3.9-5.0 mg; dissolved Fe, 0.24-0.27 mg; Mn, 0.06-0.11 mg; Cu, <0.024 mg; dissolved Zn^{2+}, <0.018 mg; CO_3^{2-}, <1.8 mg; NO_3^-, 4.37-5.93 mg; P_2O_5, 0.374-0.583 mg, and Cl^-, 9.4-11.4 mg. Salt contents in the samples were correspondent with conductivity values from 151 to 168 μSiemens cm^{-1} at 20ºC, and pH values were from 7.48 to 7.83. Distilled water had no organic matter and trace mineral ions: at 20ºC, conductivity was ≤ 20 μSiemens cm^{-1}, and pH value was 7.

2.3.2.3 Preparation of water microcosms

River water samples and distilled water were sterilized, and used for microcosm preparation and inoculation with either of the strains IVIA-1602.1 or IPO-1609 at $5x10^6$ CFU mL^{-1} similarly to Álvarez *et al.* (2008). Microcosms were incubated at 4±1ºC, 14±1ºC and 24±1ºC for 40 days. The temperature of 4ºC was selected because it had induced the VBNC state in *R. solanacearum* in pure water (van Elsas *et al.*, 2001; van Overbeek *et al.*, 2004). The temperatures of 14ºC and 24ºC were within the range in which *R. solanacearum* had been detected in environmental waters in Spain (Palomo *et al.*, 2002; Caruso *et al.*, 2005).

2.3.2.4 Cell counts

Sampling from each microcosm was at inoculation time and at 1, 2, 4, 8, 14, 28 and 40 days post-inoculation (dpi) as described (Álvarez *et al.*, 2008), to perform (i) microscopic counts of total and viable *R. solanacearum* cells by a Direct Viable Count method (Kogure *et al.*, 1979), extended to 16 h (van Elsas *et al.*, 2001) and subsequent staining with acridine orange (Oliver, 1987), and (ii) plate counts of culturable cells on two media, the

general YPGA and the semiselective medium South Africa (SMSA) agar developed for *R. solanacearum* isolation (Elphinstone *et al.*, 1996). Both media are recommended by EU Directives to isolate the pathogen from environmental samples (Anonymous, 1998, 2006).

2.3.2.5 Cell morphology

Bacterial cell shape was observed by direct staining of *R. solanacearum* cells with acridine orange, according to Álvarez *et al.* (2008). To confirm changes in typical cell morphology, specific immunofluorescence staining with the polyclonal antiserum 1546-H IVIA against *R. solanacearum* (Caruso *et al.*, 2005) was also performed. At each sampling time and for each temperature the number of bacilli and/or cocci from at least 20 random fields was counted (approximately 300 cells).

2.3.2.6 Pathogenicity assays

Ability of starved *R. solanacearum* cells incubated in the river water microcosms at 4ºC, 14ºC and 24ºC to induce disease was tested from each triplicate microcosm at each sampling time on groups of 14 tomato plants cv. Roma aged three weeks by stem inoculation of volumes of 10 μL directly taken from the microcoms. Plant inoculations were carried out according to EU Directives (Anonymous, 1998, 2006). Positive and negative controls were performed on groups of 14 plants by injecting 10 μL of a freshly growing cell suspension of either the Spanish or Dutch *R. solanacearum* strain, or PBS, respectively. Incubation of the plants under quarantine conditions, monitoring of disease symptoms, and isolation and identification of the pathogen from the wilting plants were done as described (Álvarez *et al.*, 2008).

2.3.2.7 Statistical analyses

Survival assays were performed at three incubation temperatures independently with each of the four environmental water samples and distilled water in triplicate *R. solanacearum*-inoculated microcosms. Total, viable and culturable data of *R. solanacearum* cell counts were log-

transformed, and mean values analysed by a linear regression model considering the following factors: incubation temperature, type of water (environmental or distilled), water sample (among the four of them), period of incubation, media, and bacterial strain. Differences among means of coccoid percentages at the three temperatures were estimated by variance analysis (ANOVA). A P value < 0.05 was defined as significant.

2.3.3 Results

2.3.3.1 Combined effect of temperature and nutrient limitation on *R. solanacearum* survival in water microcosms

At low (4ºC) temperature, in environmental and distilled water (Fig. 2.3.1, one representative water sample), total populations of the Spanish strain of *R. solanacearum* remained above their initial inoculation numbers throughout the 40-day experiments, while viability was slightly lower, with declines approximately from 25-30 dpi in both types of water. In contrast, culturable bacterial populations significantly decreased ($P < 0.05$) about one log unit up until eight and four dpi for river and distilled water respectively, pointing out a proportion of cells sensitive to low-temperature conditions. Thereafter progressively and significant stronger losses in culturability occurred, with values below detection level (10^1 CFU mL^{-1}) by 40±7 and 20±3 days, depending on the water sample, in river and distilled water respectively (Fig. 2.3.1). These drops in culturable counts with high numbers of cells still viable indicated a majority of the populations becoming VBNC. The Spanish strain displayed similar trends in the microcosms of the other river water samples at 4ºC, only with differences in non-culturability between environmental and distilled water ($P < 0.05$). YPGA and SMSA media yielded similar results for each of the water samples ($P > 0.05$).

At temperate (14ºC) and warm (24ºC) temperatures, trends in total, viable and culturable populations were similar ($P > 0.05$), and so only those

at 24ºC have been plotted in Fig. 2.3.1. At both temperatures, total populations of the Spanish strain remained above 10^7 cells mL^{-1} in environmental water and around this value in distilled water, and viability was slightly lower in both types of water for the 40-day experiments. During the period, culturability kept roughly at 10^7 CFU mL^{-1} in environmental water while in distilled water culturable cells stabilized below this value (Fig. 2.3.1). Assays with the other water samples yielded analogous results, also on both media ($P > 0.05$).

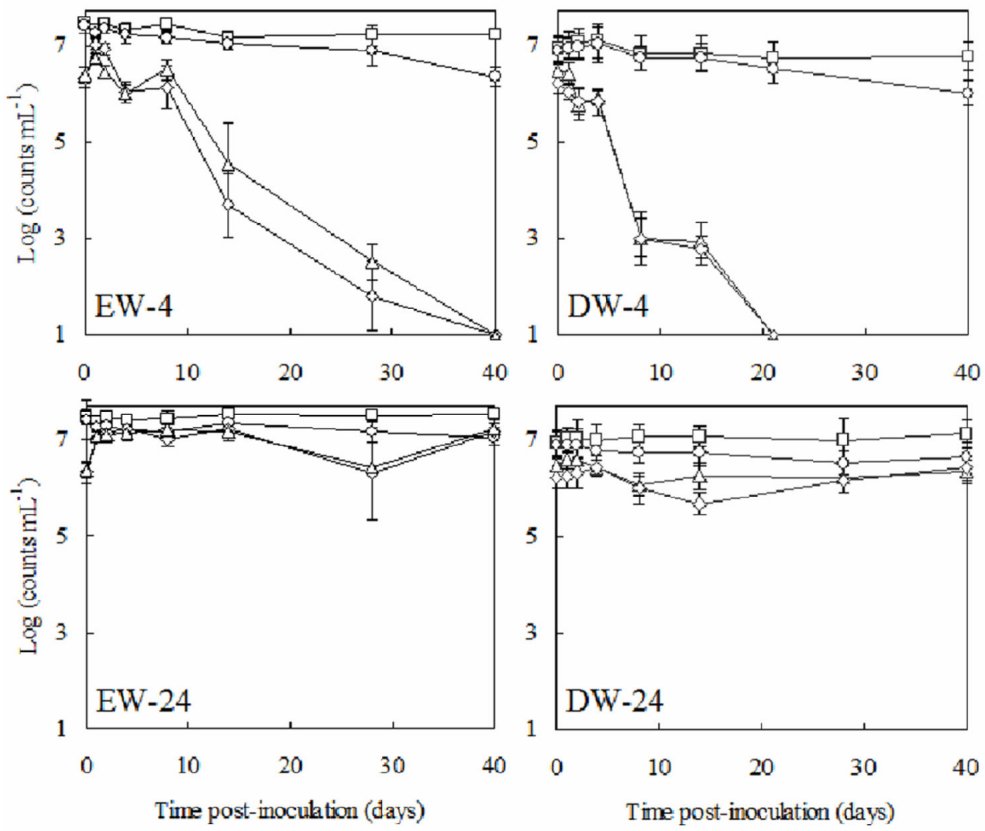


Figure 2.3.1. Effect of low temperature under nutrient-limiting conditions on survival of *R. solanacearum* strain IVIA-1602.1 during 40-day periods in water. Microcosms of: EW-4, environmental water at 4ºC; DW-4, distilled water at 4ºC; EW-24, environmental water at 24ºC, and DW-24, distilled water at 24ºC. Total (■), viable (●), and culturable cells on SMSA (▲) and YPGA (♦) media. Data from one representative environmental water sample have been plotted. Points are mean ± standard deviation of triplicate microcosms.

Similarity in trends of culturable data from the microcosms of the four water samples inoculated with the *R. solanacearum* strains could be observed by the statistical analyses. For comparative purposes, increments of culturable data at 4ºC and 24ºC were jointly calculated with respect to the initial value and plotted within time to assess the effect of water sample (Fig. 2.3.2, left), and the effect of media (Fig. 2.3.2, right). Similarity in trends was also observed after calculation of increments of culturable data at 4ºC and 14ºC, and of total and viable data at the three temperatures (not shown).

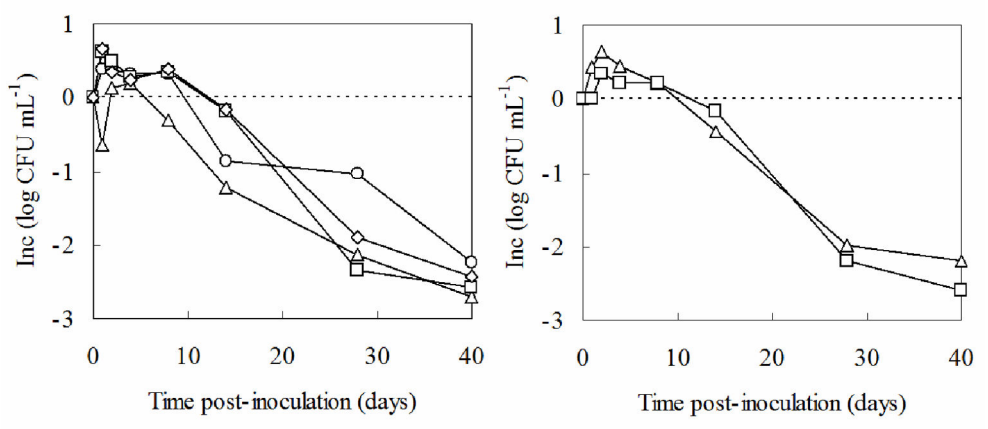


Figure 2.3.2. Similarity in trends of culturable cell counts from *R. solanacearum* strain IVIA-1602.1-inoculated environmental water microcosms throughout 40-day periods at 4ºC and 24ºC. Inc stands for Increments, which were calculated with the mean values of culturable data at both temperatures with respect to the initial ones. (Left): comparison of culturable data on SMSA medium among environmental water (EW)-1 (♦), EW-2 (●), EW-3 (▲), and EW-4 (■). (Right): comparison between culturable data on SMSA (■), and YPGA (▲) media for the four environmental water samples.

Population dynamics of total, viable and culturable cells of the Dutch *R. solanacearum* strain were similar to those of the Spanish strain ($P > 0.05$) in triplicate microcosms from both types of water and the four river water samples (not shown).

2.3.3.2 Combined effect of temperature and nutrient limitation on *R. solanacearum* cell morphology in environmental water microcosms

Cells of the Spanish strain were examined in triplicate microcosms from each of the four water samples with similar results ($P > 0.05$). Data from one representative of them are plotted in Figure 2.3.3.

At low (4ºC) temperature and throughout the 40-day experiments, bacterial cells showed the typical *R. solanacearum* bacillar morphology and coccoid cells were seldom detected, with a frequency <1% depending on the water sample (Fig. 2.3.3).

At temperate (14ºC) temperature, a great majority of *R. solanacearum* cells kept bacillar shape. Coccoids were observed in a low proportion, with constant percentages around a value between 1-3% throughout the first 28 days, and then a slight increase up to values ranging 2-6% by 40 dpi, depending on the water sample (Fig. 2.3.3).

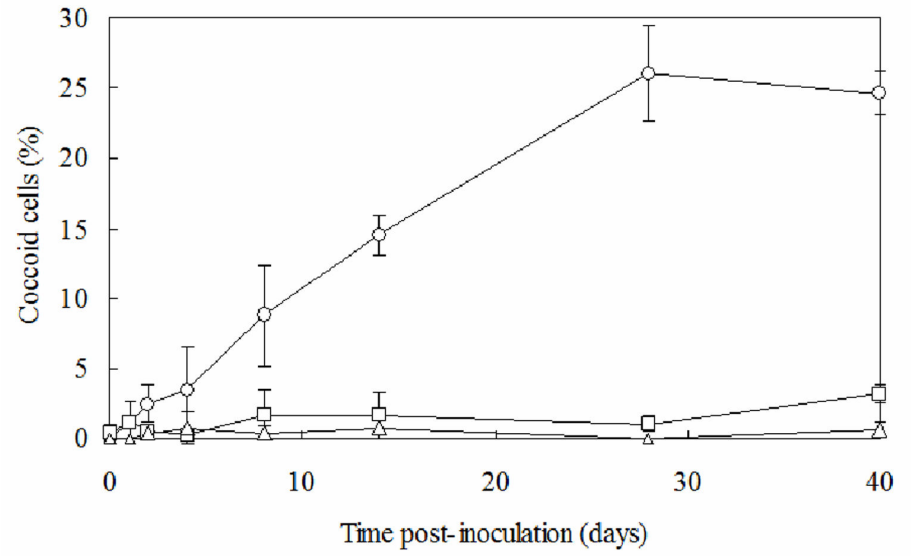


Figure 2.3.3. Proportions of coccoid cells appearing in *R. solanacearum* strain IVIA-1602.1 populations starved in the environmental water microcosms during 40-day periods at 24ºC (●), 14ºC (■), and 4ºC (▲). Data from one representative environmental water sample have been plotted. Points are mean ± standard deviation of triplicate microcosms.

At warm (24ºC) temperature, *R. solanacearum* bacilli remained a majority but, coccoid cells were more frequent: depending on the water sample, percentages by the first week were around 8-10%, progressively increasing to 13-16% by the second week, and then up to 22-32% by 28 dpi which stabilized to the end of the 40 days (Fig. 2.3.3).

Among the three temperatures, the average percentage of coccoids significantly increased with temperature ($P < 0.05$). Cell shape of the Dutch *R. solanacearum* strain showed the same trends at each of the three temperatures (not shown).

2.3.3.3 Combined effect of temperature and nutrient limitation in environmental water microcosms on *R. solanacearum* pathogenicity

During the 40-day periods in the microcosms, plant-inoculated *R. solanacearum* cells at 4ºC induced disease in 98-100% of the tomato plants (Fig. 2.3.4, one representative water sample); similar wilting percentages were obtained with cells from microcosms at 14ºC and at 24ºC (Fig. 2.3.4), and were comparable to those of the Dutch strain (not shown). At the three temperatures and depending on the water sample, viable *R. solanacearum* cells inoculated per plant were about 10^5 throughout the 40-day sampling periods, and only from approximately 28 dpi at 4ºC there was a slight decline to values around 10^4 viable cells per plant (Fig. 2.3.4). Culturable cells at 4ºC inoculated per plant ranged 10^5-10^4 in the initial dpi depending on the water sample, then they were decreasing until 10^4-10^3 CFU per plant by the first week, and progressively to <10 CFU per plant by 28 dpi and to undetectable levels by 40 dpi; at 14ºC and 24ºC culturable cells were 10^5-10^4 per plant throughout the sampling periods (Fig. 2.3.4). Plants started to show symptoms within 8-11 dpi and completely wilted within four weeks. The pathogens were re-isolated on SMSA agar from the diseased plants and PCR-identified. Negative control plants did not show any symptoms.

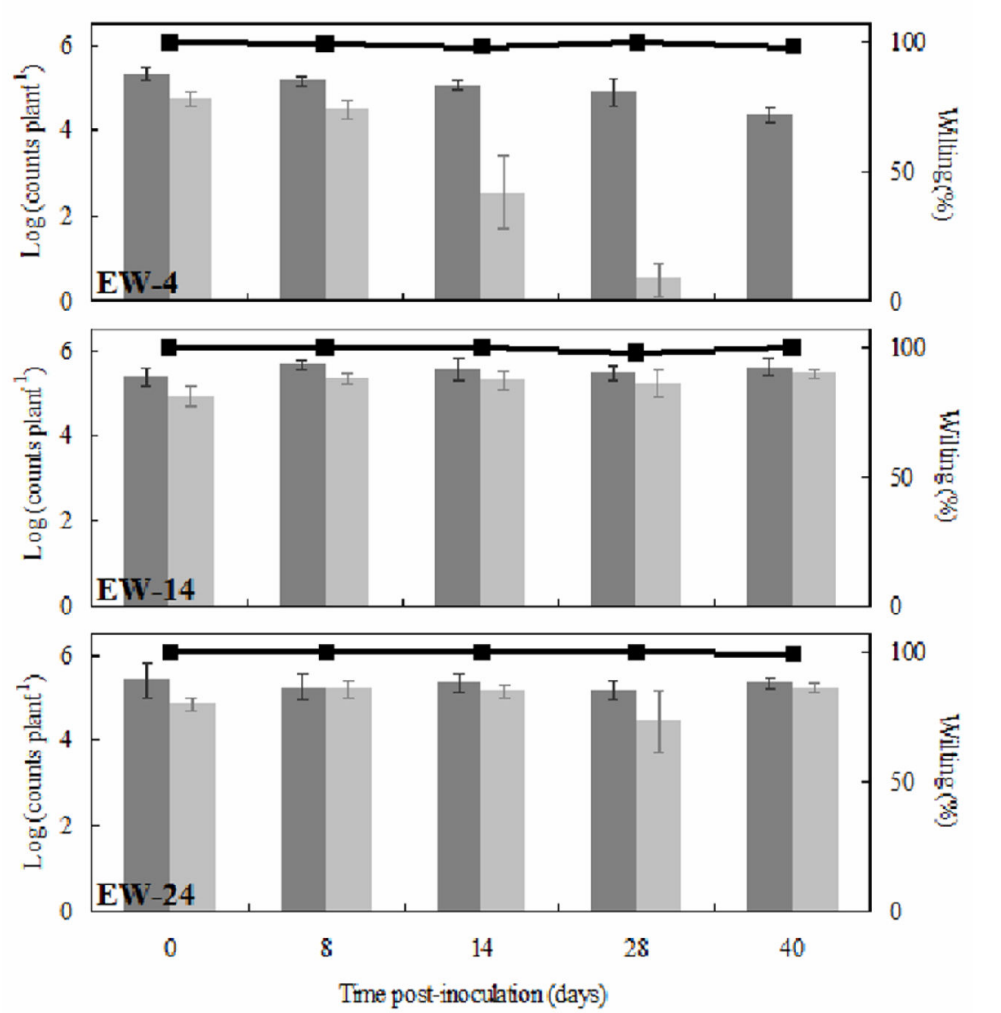


Figure 2.3.4. Pathogenicity of *R. solanacearum* strain IVIA-1602.1 starved in environmental water microcosms during 40 days, on tomato plants cv. Roma. Viable (dark grey bars) and culturable (pale grey bars) cells per plant, and percentage of wilted plants (■). Only assays performed in at least weekly intervals from one representative environmental water (EW) at 4ºC (EW-4), 14ºC (EW-14) and 24ºC (EW-24) have been plotted. Points are mean ± standard deviation (SD) of triplicate microcosms. Absolute value for 100% wilting refers to 42 plants (14x3 sets). At the three temperatures, SD of wilting values for most of the points was zero, and ± 2.0 % in some cases. Control plants inoculated with freshly grown *R. solanacearum* strain IVIA-1602.1 yielded 100% wilting, while those inoculated with PBS were negative.

2.3.4 Discussion

Distinct adaptations by *R. solanacearum* ph II bv 2 from different habitats exposed to various environmental temperatures in oligotrophic freshwaters for 40 days were observed, which allowed the pathogen to survive without loosing capacity to wilt.

At low temperature viable *R. solanacearum* ph II bv 2 populations adapted to coldness by progressively being induced into a VBNC state in environmental waters. Previous work in the literature described a loss in culturability of the pathogen under low temperature in natural water, but without assessing viability of the populations, and so without confirming VBNC cells (Kelman, 1956; van Elsas *et al.*, 2001). As the VBNC state constitutes a physiological state distinct from that of normal growth (Heim *et al.*, 2002) activated in response to environmental stress (Oliver, 2005), exposure of *R. solanacearum* to the temperature of 4ºC, known to prevent multiplication of the pathogen (Kelman, 1953), proves to be stressful for the general fitness of the bacterium. The fact that cold-adapted water bacteria are not likely to be cold-induced VBNC (Vattakaven *et al.*, 2006), contrarily to what observed in this work with *R. solanacearum* from different climates, suggests that ph II bv 2 strains of the pathogen are not naturally cold-adapted, even those isolated from cold habitats.

Since the process was more slowly in environmental waters, the VBNC *R. solanacearum* cold-induction period seemed to be dependent on water nutrient contents, namely trace organic matter and some dissolved salts available for the cells in environmental waters but absent in distilled water, and so nutrient concentrations not supporting *R. solanacearum* growth could act as an additional stress contributing to the cold-induction of the VBNC state. Likewise, a lower mineral salt concentration markedly shortened the VBNC *Vibrio parahaemolyticus* induction period (Wong & Wang, 2004). In ultrapure water, *R. solanacearum* population dynamics during the VBNC entrance by low temperature had been also monitored for the Dutch ph II bv 2 strain, but culturability was tested differently (van Overbeek *et al.*, 2004). Bacterial species as *V. vulnificus* and *Aeromonas hydrophila* behaved similarly to *R. solanacearum* under low-temperature and

nutrient-limiting conditions (Biosca *et al.*, 1996; Mary *et al.*, 2002), whilst others such as *Campylobacter jejuni* and *Erwinia amylovora* displayed different responses (Rollins & Colwell, 1986; Biosca *et al.*, 2006).

At temperate and warm temperatures, *R. solanacearum* ph II bv 2 populations remained readily culturable, displaying starvation-survival responses faced to nutrient limitation in environmental waters as described at 24ºC (Álvarez *et al.*, 2008) and similar in terms of population levels. Lack of unculturability was in agreement with previous work reporting isolation and favourable persistence of the pathogen in environmental waters at temperatures allowing its multiplication (Wenneker *et al.*, 1999; van Elsas *et al.*, 2001; Caruso *et al.*, 2005; Hong *et al.*, 2005). Furthermore, the existence of organic matter and salts in environmental waters contributed to stimulate *R. solanacearum* survival. Presence of trace minerals in comparison to their absence also facilitated culturability of *Aerobacter aerogenes* (Trulear & Characklis, 1982) and *Leuconostoc mesenteroides* (Kim *et al.*, 2000) since mineral salts can affect not only cell growth but also cell survival during nutrient limitation conditions (Morita, 1997).

Morphological changes are a visible indicator of adaptation to the environment (Morita, 1997; Shi & Xia, 2003). Transformation of starved *R. solanacearum* ph II bv 2 cells from the normal bacilli into coccoids, in different proportions according to temperature, was an adaptation to oligotrophy since shape rounding off and size reduction allow nutrients be sequestered more efficiently (Morita, 1997). Although cells entering the VBNC state often exhibit dwarfing (Oliver, 2005), at low temperature *R. solanacearum* coccoids were seldom observed, probably because this temperature rapidly causes stress and induces dramatic decreases in cell metabolism and uptake of water nutrients. Interestingly, at both starvation-survival-inducing temperatures the proportions of coccoids formed differed largely. Contrarily to what occurred in temperate conditions, at warm temperature a significant proportion of *R. solanacearum* cells converted into coccoids to optimize water nutrient uptake, as described (Álvarez *et al.*, 2008). Thus, transition to coccoid cells would be mostly influenced by nutrient limitation and to a lesser extent by low temperature, as reported (Klančnik *et al.*, 2008). Rather, temperature would have a role

on this adaptation to oligotrophy when within the pathogen's growth range, acting on cell metabolism rate and nutrient requirement frequency as proposed (Christophersen, 1973). Similarly to *R. solanacearum*, a number of bacterial species decreased their sizes with increasing environmental temperatures (Christophersen, 1973; Rollins & Colwell, 1986; Mary *et al.*, 2002). Notwithstanding, this can not be considered a general bacterial behaviour (Christophersen, 1973; Biosca *et al.*, 1996; Shi & Xia, 2003).

Although temperatures as 14ºC and 24ºC are physiologically permissive for survival and multiplication of *R. solanacearum* ph II bv 2 strains introduced to temperate areas (growth range in nutrient-rich laboratory conditions: 8-10°C to 37-39°C, optimum: 28±1ºC; Kelman, 1953), in natural nutrient-deprived environments the stress of oligotrophy would be less intense for the pathogen at temperatures around 14ºC than at values nearer to the optimum as 24ºC, provided the lack of nutrients to hold a faster energy-consuming metabolism usually imposed by higher temperatures. Moreover, in the presence of indigenous microbiota *R. solanacearum* survived longer at 14ºC than at 24ºC in oligotrophic environmental waters (Álvarez *et al.*, 2007), and culturability of the Dutch *R. solanacearum* strain was favoured at 12ºC and 20ºC rather than at 28ºC in agricultural water both in the presence and absence of other aquatic microorganisms (van Elsas *et al.*, 2001). In this work, strains isolated from either cold or warm areas were apparently better temperate-adapted than cold-adapted as considered (Brown, 2009). In consequence, within the temperature interval applied by EU legislation to transport suspected water samples (4ºC to 10ºC; Anonymous, 2006), temperatures around 10ºC would be more advisable to keep the samples than values around 4ºC since cultivation-based methods are required to confirm pathogen detection.

Regardless of the temperature the starved *R. solanacearum* ph II bv 2 was confronted with in environmental waters, the pathogen kept capacity for *in planta* multiplication and colonization, being highly virulent throughout the monitored period. Likewise, starved and/or cold-stressed and/or starvation-induced VBNC and/or cold-induced VBNC *R.*

solanacearum cells were infective and pathogenic for variable periods (van Overbeek *et al.*, 2004; Caruso *et al.*, 2005; Álvarez *et al.*, 2008).

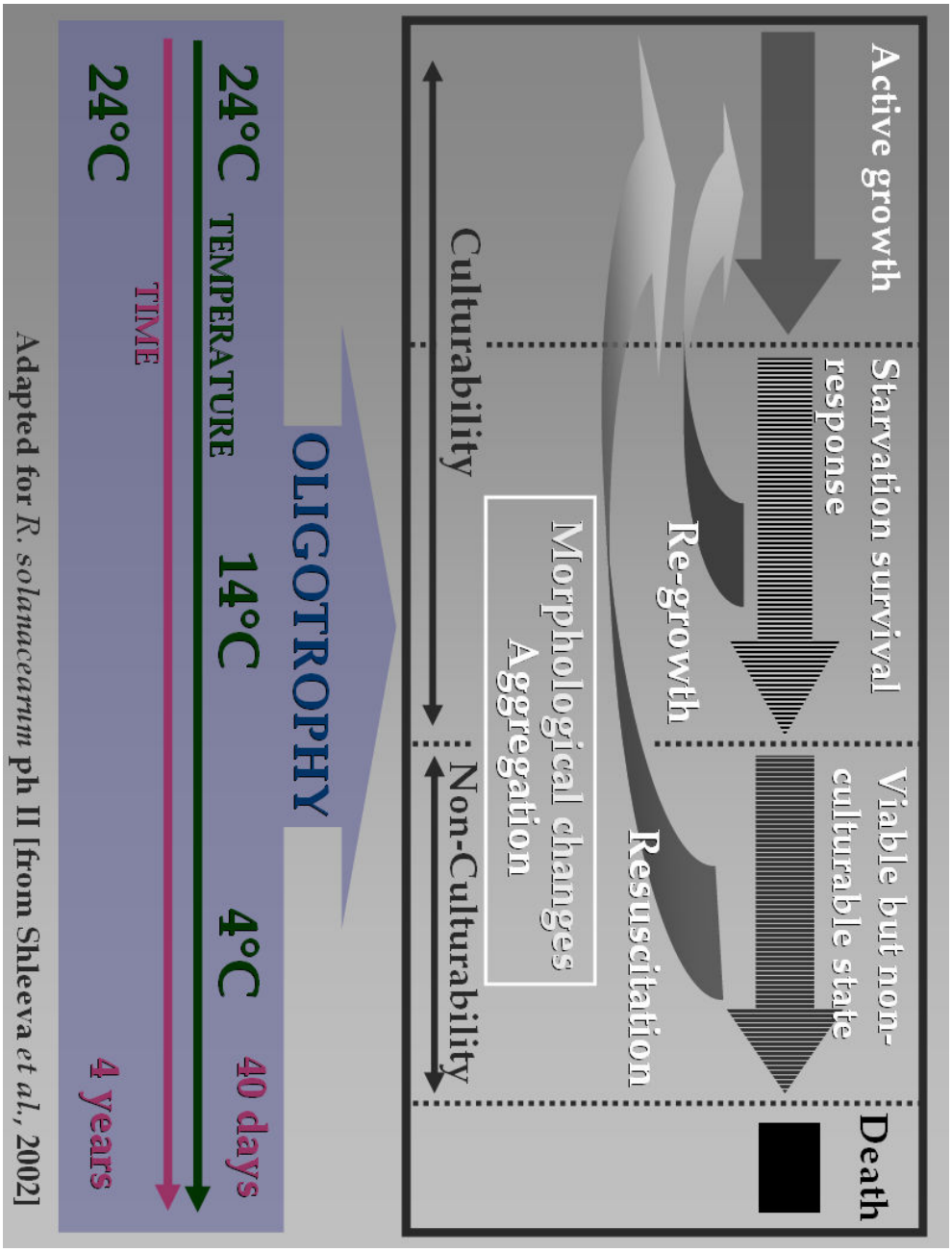
Overall, European *R. solanacearum* ph II bv 2 adapted faced to a combined effect of temperature and oligotrophy. At low temperature the delay in the induction of the VBNC state in environmental waters suggested a protective effect of water nutrient contents on bacterial cells, and pointed out the relevance of performing survival studies in conditions better approaching those of natural environments. At temperate and warm temperatures, adaptations to oligotrophy were starvation-survival responses and morphological changes influenced by temperature. It appeared that, when temperature was the main stress (cold conditions), nutrient deprivation acted as an additional stress contributing to accelerate the effect of temperature, and conversely, when oligotrophy was the main stress (temperate and warm conditions), temperature increased the effect of oligotrophy. In all conditions cells remained virulent. Understanding *R. solanacearum* ph II bv 2 adaptations to environmental stresses can help to design strategies to control its dissemination in natural settings.

Acknowledgements

B Álvarez thanks the IVIA for a predoctoral grant. The authors thank JL Palomo and the Consejería de Agricultura de Castilla-León for sending water samples, JD van Elsas for strain IPO-1609, and E Carbonell, J Pérez and JL Díez for the statistical analyses. This work was funded by the EU project QLK 3-CT-2000-01598.

The next Figure (following page) has been included to summarize the main survival strategies observed for *R. solanacearum* ph II under the conditions of extended oligotrophy and oligotrophy at different environmental temperatures in water microcosms which have been applied in the experiments performed in the Chapters 2.2 and 2.3 of this work, respectively.

The scheme is based on a generic model proposed by Shleeva *et al.* (2002), and has been adapted by A. Trigalet, M.M. López, E.G. Biosca & B. Álvarez on the grounds of the results reported in these chapters. For details, see the corresponding discussions.


Active growth
Starvation survival response
Viable but non-culturable state
Death
Re-growth
Resuscitation
Morphological changes
Aggregation
Culturability
Non-Culturability
OLIGOTROPHY
24°C
TEMPERATURE
14°C
4°C
40 days
24°C
TIME
4 years
Adapted for R. solanacearum ph II [from Shleeva et al., 2002]

CHAPTER 2.4

Influence of native microbiota on the survival of *Ralstonia solanacearum* phylotype II in river water microcosms

Belén Álvarez, María M. López & Elena G. Biosca

Applied and Environmental Microbiology **(2007)**
73 (22): 7210–7217

Influence of native microbiota on the survival of *Ralstonia solanacearum* phylotype II in river water microcosms

Abstract

Ralstonia solanacearum phylotype II biovar 2 causes bacterial wilt in solanaceous hosts producing severe economic losses worldwide. Waterways can be major dissemination routes of this pathogen which is able to survive for long periods in sterilized water. However, little is known about its survival in natural water when other microorganisms such as bacteriophages, other bacteria and protozoa are present. This study looks into the fate of a Spanish strain of *R. solanacearum* inoculated in water microcosms from a Spanish river, containing different microbiota fractions, at 24ºC and 14ºC, for a month. At both temperatures, *R. solanacearum* densities remained constant at the initial levels in control microcosms of sterile river water while, by contrast, declines in the populations of the introduced strain were observed in the non-sterile microcosms. These decreases were less marked at 14ºC. Lytic bacteriophages present in this river water were involved in the declines of the pathogen populations, but indigenous protozoa and bacteria also contributed to the reduced persistence in water. *R. solanacearum* variants displaying resistance to phage infection were observed, but only in microcosms without protozoa and native bacteria. In water microcosms, the temperature of 14ºC was more favourable for the survival of this pathogen than 24ºC, since biotic interactions were slower at the lower temperature. Similar trends were observed in microcosms inoculated with a Dutch strain. This is the first study demonstrating the influence of different fractions of water microorganisms on the survival of *R. solanacearum* phylotype II released into river water microcosms.

2.4.1 Introduction

Ralstonia solanacearum Yabuuchi *et al.* (Smith) (Yabuuchi *et al.*, 1995) is a devastating plant pathogenic bacterium that produces severe economic losses in many crops worldwide (Elphinstone, 2005; Hayward, 1991). It is considered a quarantine organism according to European legislation (Anonymous, 1998, 2000, 2006) and a potential bioterrorism agent in the U.S.A. (Madden & Wheelis, 2003). The species is classified into races and biovars (Hayward, 1991) and more recently in phylotypes (Fegan & Prior, 2005), according to host range, biochemical or molecular characteristics respectively. Phylotype (ph) II contains the *R. solanacearum* race 3 biovar 2 of this species (Fegan & Prior, 2005), which is the causative agent of bacterial wilt in solanaceous crops (Buddenhagen & Kelman, 1964) and some ornamental plants in temperate climates (Swanson *et al.*, 2005).

In Europe, this pathogen was first detected in Swedish river water (Olsson, 1976) and, in the last ten years, it has been reported in many West European countries (Elphinstone, 2005; Elphinstone *et al.*, 1998; Janse, 1996; Janse & Schans, 1998; Marquínez & Noval, 1996). The origin of most of these outbreaks has been associated to irrigation with contaminated water (Elphinstone *et al.*, 1998; Janse & Schans, 1998; López & Biosca, 2005; Olsson, 1976), where the pathogen can persist as a free-living form and/or in roots of the riparian weed *Solanum dulcamara* or other plants (Coutinho, 2005; Elphinstone, 2005; Elphinstone *et al.*, 1998; López & Biosca, 2005).

In spite of the fact that *R. solanacearum* ph II is relatively frequently detected in watercourses in Europe (Caruso *et al.*, 2005; Elphinstone, 1996; Elphinstone *et al.*, 1998; Janse & Schans, 1998) and more recently also in pond water in the U.S.A. (Hong *et al.*, 2005), its fate in aquatic environments, where the pathogen can be affected by several abiotic factors (van Elsas *et al.*, 2001, 2005), is still poorly understood (Coutinho, 2005; López & Biosca, 2005; van Elsas *et al.*, 2005). In experimental microcosms, *R. solanacearum* is able to survive for long periods in sterile water under nutrient starvation at moderate temperatures (Kelman, 1956; van Elsas *et al.*, 2001) retaining its pathogenicity (Caruso *et al.*, 2005; van Overbeek *et al.*, 2004). However, its survival is negatively affected by extreme temperature

values (van Elsas *et al.*, 2001; van Overbeek *et al.*, 2004). In fact, field studies have shown that temperature is a key factor influencing the populations of this bacterium in water (Caruso *et al.*, 2005; Elphinstone *et al.*, 1998; Janse & Schans, 1998). A correlation between *R. solanacearum* populations in river water and water temperature has been demonstrated, with naturally starved cells of this pathogen maintaining their pathogenicity (Caruso *et al.*, 2005).

The fate of this pathogen in environmental waters may also depend on the presence of native microorganisms, reported to be involved in predatory, competitive or parasitic events in other bacterial models (Flint, 1987; González *et al.*, 1992; Guerrero *et al.*, 1986; Marco-Noales *et al.*, 2004; McCambridge & McMeekin, 1980; Sime-Ngando *et al.*, 2003). Within the range of water microbiota, bacteriophages and protozoa represent a relatively constant source of mortality for bacterial populations in watersystems (Bettarel *et al.*, 2004; Guixa-Boixereu *et al.*, 1999; Gurijala & Alexander, 1990; Marco-Noales *et al.*, 2004; Vrede *et al.*, 2003; Weinbauer, 2004). For instance, in river water, the disappearance of *Escherichia coli* has been related to the presence of bacteriophages and bacteria (Flint, 1987), as well as to predatory protozoa (Arana *et al.*, 2003; González *et al.*, 1992). In the same way, a strong effect of indigenous microbiota on the survival of *R. solanacearum* in agricultural drainage water microcosms has been reported (van Elsas *et al.*, 2001) but the nature and influence of the main groups of the aquatic biota were not investigated.

Our research was motivated by the scarce information available on the fate of *R. solanacearum* in natural water, together with the fact that this pathogen had been detected in some watercourses in Spain (Caruso *et al.*, 2005; Palomo & García-Benavides, 2002). The objectives were to establish whether *R. solanacearum* ph II can survive in microcosms of natural water from a Spanish river and to ascertain the influence of aquatic microorganisms on its survival, which is related to the persistence of the bacterium in the environment. River water microcosms inoculated with relatively high populations of this pathogen were selected as a model that would be mimicking what would be happening in environmental watersystems when occasionally polluted by *R. solanacearum*.

2.4.2 Material & Methods

2.4.2.1 Bacterial strains and growth conditions

R. solanacearum ph II strain IVIA-1602.1 (race 3 biovar 2) isolated from potato in Spain was used. Furthermore, in some experiments, the Dutch strain IPO-1609 ph II (race 3 biovar 2) kindly provided by J. D. van Elsas, or a variant of the Spanish strain resistant to river water phages (isolated during the survival experiments), were included for comparative purposes. This resistant variant was identified by biochemical, serological and molecular methods (Anonymous, 1998, 2006). Strains were kept at –80 ºC in a 30% (v/v) glycerol medium. They were grown on the non-selective Yeast Peptone Glucose Agar medium (YPGA) (Lelliot & Stead, 1987) for 72 h at 29ºC.

2.4.2.2 Water samples

Water samples were collected from the river Tormes (Northwestern Spain) in three sampling sites contaminated with *R. solanacearum* (Caruso *et al.*, 2005; Palomo & García-Benavides, 2002), as described by Caruso *et al.* (2005).

Protozoa were counted in each water sample after formaldehyde 2% (v/v) fixation. Samples were filtered through a 100 μm pore size sieve and the particles between 30 and 100 μm were counted by a Coulter Z Series device (© Coulter Corporation). After staining by acridine orange (Oliver, 1987), microscopic observation of the samples was also performed by epifluorescence to confirm the presence of protozoa and to discard protophyta. A microscope Nikon Eclipse E800 was used at magnifications of x400 and x600.

Culturable counts of native bacteria and *R. solanacearum* were respectively performed on YPGA, and a modified Semiselective Medium South Africa (SMSA) agar (Elphinstone *et al.*, 1996) after incubation for 72 h at 29ºC. Semiselectivity for *R. solanacearum* is mainly based on the action of four antibiotics (penicillin, polymyxin, chloramphenicol and bacitracin),

triphenyl-tetrazolium chloride and crystal violet (Anonymous, 1998, 2006; Elphinstone *et al.*, 1996). Identification of putative *R. solanacearum* water isolates and biovar characterization were performed as abovementioned (Anonymous, 1998, 2006).

Bacteriophage enrichment detection assays for *R. solanacearum* lytic phages were performed in triplicate by adding 1 ml aliquots of each 0.22 μm filtered river water sample to log-phase cultures of *R. solanacearum* cells (about 10^9 CFU/ml) in 10 ml and 5 ml of a modified Wilbrink broth (Caruso *et al.*, 2002), according to Hendrick and Sequeira (Hendrick & Sequeira, 1984). Bacterial suspensions without filtered water were used as negative controls. Incubations were done in flasks of 50 ml with shaking (165 r.p.m.) at 29ºC and 35ºC, because both temperatures had previously been used to improve the recovery of *R. solanacearum* in water samples from this river (Caruso *et al.*, 2005). The cleared suspensions (lysates) were tested for phage enumeration on YPGA plates according to a standard surface plating method (Civerolo, 1990). Based on this kind of enrichment assays, the initial concentrations of lytic phages of *R. solanacearum* in the river water were estimated according to the Most Probable Number (MPN) technique (Weinbauer, 2004), by determining the number of tubes in each group that became cleared and testing for presence of plaques.

2.4.2.3 Survival experiments in river water microcosms

Natural river water microcosms were prepared with water samples as described in other models (Flint, 1987; Marco-Noales *et al.*, 2004). Briefly, four types of microcosms were prepared in duplicate for each of the three water samples used in each of the survival experiments: (i) untreated river water, with the whole microbiota; (ii) 0.8 μm filtered water, to keep most protozoa apart; (iii) 0.2 μm filtered water, to remove most of bacteria and (iv) control microcosms, with 0.2 μm filtered and autoclaved water to inactivate virus. *R. solanacearum* strain IVIA-1602.1 was inoculated at a final concentration of 0.5% ($5x10^6$ CFU/ml) in a volume of 100 ml for each microcosm. Samples were incubated in the dark in the static state at either

24±1ºC or 14±1ºC for one month. Both temperatures were selected because they were within the range in which *R. solanacearum* had been detected in this river (Caruso *et al.*, 2005; Palomo & García-Benavides, 2002).

For some experiments, supplementary microcosms prepared as abovementioned were also inoculated with either the Dutch strain IPO-1609, or a variant of the Spanish strain resistant to river water phages isolated during the experiments (from 0.2 μm filtered water microcosms), as described below.

2.4.2.4 Total, viable and culturable *R. solanacearum* cell counts

Sampling from the microcosms was initially performed at inoculation time (day 0) and 3, 6, 9, 15, 21 and 33 days post-inoculation (dpi) in preliminary assays and at 0, 1, 2, 4, 8, 15, 22 and 28 dpi in subsequent experiments. Plate counts were done in duplicate, after incubation for 72 h at 29ºC on YPGA and SMSA media, with a detection limit of about 10 CFU/ml. Total and viable cells were microscopically counted by a Direct Viable Count (DVC) modified method (Kogure *et al.*, 1979; van Elsas *et al.*, 2001). According to this method, yeast extract and nalidixic acid were added to aliquots to reveal the presence of viable cells, which elongated without dividing. Subsequently, cells were stained by acridine orange as described (Oliver, 1987). For microcosms with indigenous bacteria, *R. solanacearum* cell staining was performed by indirect immunofluorescence (IF) (Anonymous, 1998, 2006) with a polyclonal antiserum 1546-H IVIA (Caruso *et al.*, 2005). The detection limit of this technique is around 10^3 cells/ml (Anonymous, 1998, 2006) but it was ten-fold increased for some samples by processing higher volumes. Cells were visualized with a Leika epifluorescence microscope at an amplification of x1250.

2.4.2.5 Co-culture of *R. solanacearum* strain IVIA-1602.1 and a purified river water phage

To confirm the influence of indigenous river water phages on *R. solanacearum* populations, sterile river water was co-inoculated with the strain IVIA-1602.1 at about 10^6 CFU/ml (similar to that inoculated in river water microcosms) and the selected phage at $2x10^2$, $2x10^3$, $7x10^3$ or $2x10^4$ PFU/ml (within the estimated range for river water by the MPN method) at 24ºC. Similar bacterial concentrations and phage at $2x10^3$ and $7x10^3$ PFU/ml were co-inoculated and monitored at 14ºC. Sterile river water with either *R. solanacearum* or the phage was used as a control. Aliquots for bacteria and phage counts were taken regularly during 24 h at 24ºC or 48 h at 14ºC, serially ten-fold diluted in PBS and plated on YPGA. For phage enumeration, aliquots were previously filtered through 0.22 μm pore size membranes and plated as abovementioned (Civerolo, 1990). Total bacterial counts were performed as described above, in some of the assays at both temperatures. All the assays were done in duplicate, in separate experiments.

2.4.2.6 Statistical analysis

Each survival experiment was done at least in duplicate in independent experiments and data of total, viable and culturable *R. solanacearum* cell counts were analysed by using mean values of log-transformed data from duplicate samples. The null data from culturable counts below the detection limit were not included for the statistical analysis. Significant differences were assessed by variance analysis (ANOVA). Factors considered for the analysis were: day, media, experiment, water treatment and incubation temperature. Differences were recorded as significant at p below 0.05.

2.4.3 Results

2.4.3.1 Analysis of river water samples

Protist counts in the water samples ranged from 10^1 to 10^2 individuals/ml. Protozoa were small sized (between 30 and 45 μm) in a range of 80-95%, and also medium sized (between 45 and 60 μm) up to 20%, depending on the water sample. No protozoa longer than 60 μm were detected. Microscopic observations were in accordance with these results.

Culturable counts of river water bacteria on YPGA (from samples taken at water temperatures above 14ºC) were similar in all water samples analysed (10^3 CFU/ml), while counts for *R. solanacearum* populations on SMSA agar ranged from 15 to 45 CFU/ml. When water temperature dropped below 14ºC, river water bacteria counts decreased to 10^2 CFU/ml and the pathogen was not detected by direct isolation. For survival experiments, only water samples taken at temperatures ≥14ºC were used.

Lytic phages of *R. solanacearum* were detected and isolated from water samples, but only from those where the pathogen was recovered on SMSA agar (at ≥14ºC). Initial concentrations of these lytic phages in the river water samples were estimated between 10^2 and $>10^3$ lytic viral particles/ml. In the assays for phage detection at 29ºC, suspensions were observed to clear after overnight incubation for a 10:1 (v/v) bacterium:phage ratio and earlier for a ratio of 5:1 (v/v) in all water samples, while control suspensions became more turbid. Phage enumeration on YPGA plates spread-inoculated with fresh cultures of *R. solanacearum* yielded plaques which were visible after 36-48 h of incubation at 29ºC and continued expanding on plates up to 72 h. Plaque counts were similar in all cases (10^8-10^9 PFU/ml). Plaques were usually round with irregular edges and transparent, and some variation in size was observed. In contrast, at 35ºC, phage detection assays were negative for the same water samples that had proved positive at 29ºC. Characterization of lytic activity by selected river water phages against *R. solanacearum* strains IVIA-

1602.1 and IPO-1609 in modified Wilbrink broth confirmed such activity at 29ºC but not at 35ºC.

2.4.3.2 Survival of *R. solanacearum* strain IVIA-1602.1 in river water microcosms

Preliminary survival assays monitoring *R. solanacearum* culturability in river water microcosms at 24ºC were done for three water samples with similar results. In sterile river water, culturability of the strain was maintained at 10^7 CFU/ml, while in all microcosms with indigenous microbiota, the strain was not detected on plates from the second sampling day, at three dpi. From that time, in 0.2 μm filtered water microcosms, colonies appeared on YPGA and SMSA media which were slightly different from those of the inoculated strain and were identified as *R. solanacearum* (Anonymous, 1998, 2006). These colonies were comparably smaller, less fluid and with irregular edges, and were furtherly proved to be phage-resistant. Regarding 0.8 μm filtered and untreated water microcosms, only indigenous bacteria were observed from three dpi on YPGA (10^5 CFU/ml) and SMSA agar (10^2-10^3 CFU/ml) and no colonies of phage-resistant variants were detected. The declines in the inoculated *R. solanacearum* populations in all the non-sterile microcosms suggested the involvement of river water microorganisms. Similar results were obtained for the other two water samples.

To confirm these initial results, another three water samples taken from the same river sites were used to prepare new microcosms, which were monitored in more detail at 24ºC and 14ºC. Fig. 2.4.1 shows survival curves of strain IVIA-1602.1 in the different microcosms, for one representative water sample. Trends in the dynamics of total, viable and culturable *R. solanacearum* cells were similar for the other two water samples, showing significant differences ($p < 0.05$) between the two incubation temperatures.

Survival at 24ºC

In sterile control microcosms at 24ºC, total and viable cell counts of *R. solanacearum* remained at about 10^7 cells/ml, while culturable counts on

YPGA and SMSA plates showed a slight increase from 10^6 to 10^7 CFU/ml within one dpi and then remained constant until the end of the experiment (Fig. 2.4.1 A). By contrast, in microcosms with water biota, a significant decline ($p < 0.05$) in total, viable and culturable cell counts was observed within the first days (Fig. 2.4.1 B, C and D), as a result of the reduction of the inoculated population. With respect to 0.2 μm filtered water microcosms (Fig. 2.4.1 B), a slight decline in total and viable cell counts until approximately 10^6-10^5 cells/ml was observed within the first two days, which stabilized at these levels. The reduction in culturability was more noticeable, reaching 10^2-10^1 CFU/ml on SMSA and YPGA media at one dpi. From that day, a phage-resistant variant appeared on plates, progressively increasing to about 10^4-10^5 CFU/ml by eight dpi and stabilizing, remaining around two log units lower than the inoculated population in control microcosms. The colonies of this variant were similar to those observed in the preliminary experiment.

Regarding 0.8 μm filtered and untreated water microcosms (Fig. 2.4.1 C and D), a decrease until 10^6 cells/ml within the first dpi occurred for total and viable cell counts; from one to four dpi, there was an additional decline in them. Afterwards, microscopic cell counts by IF for the 0.8 μm filtered and untreated water microcosms were below the detection limit. The numbers of culturable inoculated cells on both media dropped to around 10 CFU/ml within the first two days in both types of microcosms, similarly to what occurred in 0.2 μm filtered water at one dpi. Then, only river bacteria were observed on YPGA plates, which stabilized at around 10^4-10^5 CFU/ml and 10^3-10^4 CFU/ml, respectively for 0.8 μm filtered and untreated river water microcosms. The decline in total, viable and culturable *R. solanacearum* cell counts in all microcosms with aquatic microorganisms confirmed the preliminary data. Moreover, the reduction in total cell numbers in these microcosms demonstrated either lysis and/or predation of *R. solanacearum* cells.

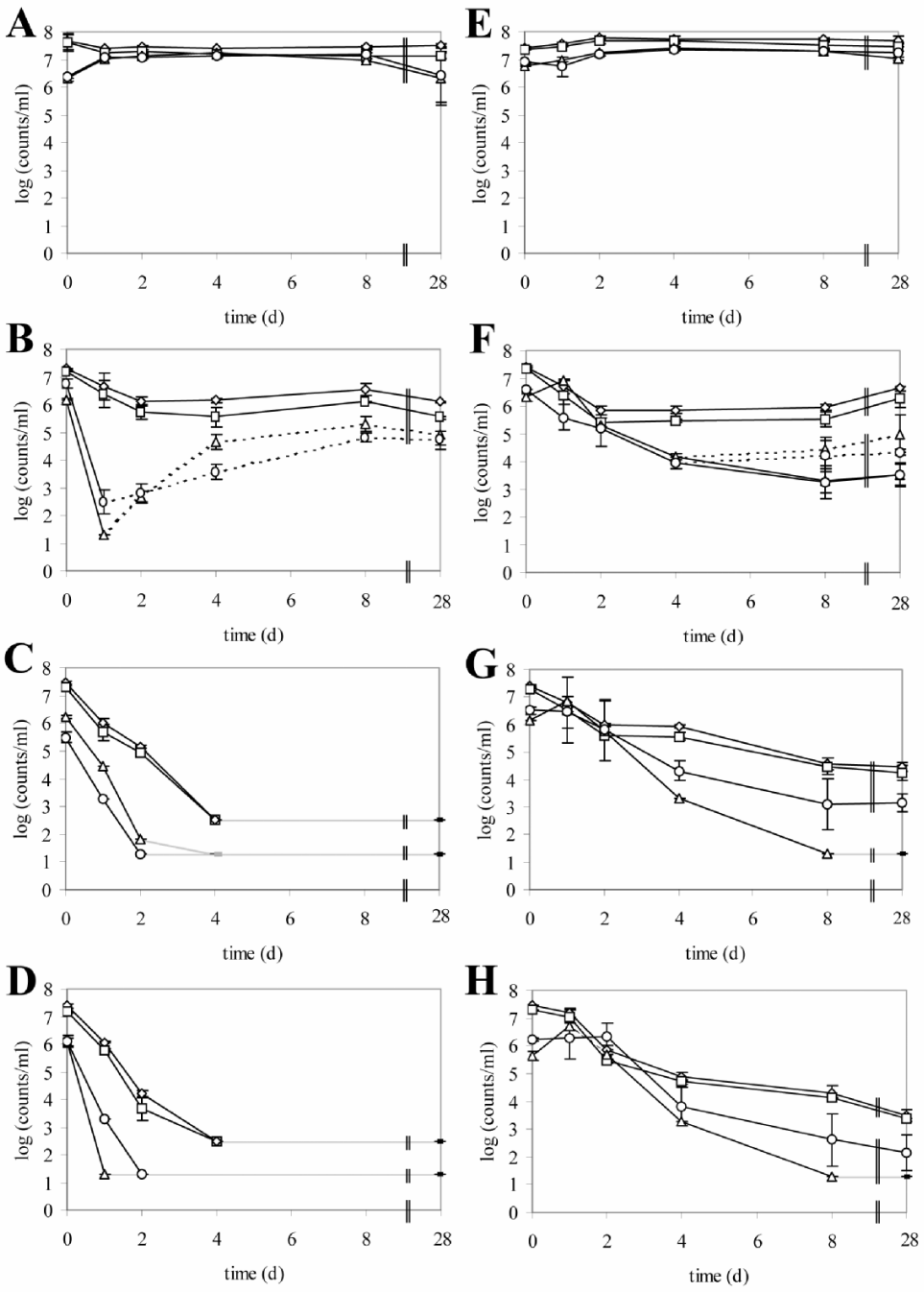


Figure 2.4.1. Dynamics of *R. solanacearum* ph II strain IVIA-1602.1: total (♦), viable (□) and culturable cell counts on YPGA (▲) and SMSA (O) plates at 24ºC (left) and 14ºC (right) in river water microcosms. (A, E) sterile control water, (B, F) 0.2 µm filtered water, (C, G) 0.8 µm filtered water and (D, H) untreated water. For 0.2 µm

filtered water microcosms, broken lines (- - -) refer to a phage-resistant variant of the inoculated strain of *R. solanacearum*. Detection threshold of the techniques were ~10^2 cells/ml for IF and ~10 CFU/ml for YPGA and SMSA. Data points for values below detection limit are shown as a dotted line. Points are the mean of two separate assays in duplicate and error bars indicate variation as standard deviation for each point. Similar results were obtained for the other two water samples.

Survival at 14ºC

Cell counts of *R. solanacearum* in sterile control microcosms at 14ºC (Fig. 2.4.1 E) were similar to those at 24ºC (Fig. 2.4.1 A), whereas the declines in total, viable and culturable cell numbers for non-sterile water microcosms showed comparably less pronounced slopes (Fig. 2.4.1: B and F, C and G, D and H). Likewise, significant differences were observed between sterile and non-sterile microcosms ($p < 0.05$). Microscopic counts in all non-sterile river water microcosms (Fig. 2.4.1 F, G and H) decreased similarly to culturability within the first two days. Afterwards, for 0.2 μm filtered water (Fig. 2.4.1 F), there was a decline in total and viable cell numbers until 10^5-10^6 cells/ml and subsequent increase which stabilized at around 10^6-10^7cells/ml. Like at 24ºC, a decrease in culturability of the inoculated strain was observed, as well as the appearance of phage-resistant colonies, although three days later (at four dpi); then, their counts increased progressively until stabilization at 10^4-10^5 CFU/ml. Unlike at 24ºC, at 14ºC colonies of the phage-sensitive strain were present simultaneously to those of the resistant but at slightly lower levels (Fig. 2.4.1 F). For 0.8 μm filtered and untreated water microcosms, *R. solanacearum* microscopic cell counts decreased progressively to about 10^5 and 10^4 cells/ml respectively, up to eight dpi (Fig. 2.4.1 G and H); culturability remained at around 10^3-10^2 CFU/ml on SMSA agar to the end of the experiment, while it dropped below detection on YPGA plates (Fig. 2.4.1 G and H). Like at 24ºC, in both types of microcosms only culturable indigenous bacteria were observed on YPGA after some days, and at similar levels (10^4-10^5 and 10^3-10^4 CFU/ml respectively).

2.4.3.3 Survival of *R. solanacearum* strain IPO-1609 in river water microcosms

To discard the possibility that the observed biotic effects only concerned the Spanish strain IVIA-1602.1, the Dutch strain IPO-1609 was also used for survival experiments in the four types of river water microcosms. Trends in the population dynamics of the Dutch strain in the microcosms were very similar to those of the Spanish strain with no significant differences up to one month, including the appearance of phage-resistant variants (data not shown).

2.4.3.4 Survival of a phage-resistant variant of strain IVIA-1602.1 in river water microcosms

To assess whether the influence of phages on *R. solanacearum* ph II survival in river water microcosms may mask to some extent other biotic effects, the four types of microcosms were inoculated with one randomly selected phage-resistant variant and its survival was monitored at 14ºC. In control river water microcosms (Fig. 2.4.2 A), total, viable and culturable counts of this variant remained slightly higher than the initial values of 10^7 cells/ml and 10^6 CFU/ml, showing a similar behaviour to that of the wild type in sterile water (Fig. 2.4.1 E). In the non-sterile microcosms different trends were observed (Fig. 2.4.2 B, C and D). In 0.2 μm filtered water (Fig. 2.4.2 B), microscopic and CFU counts confirmed the resistance of this variant to the phages present in this water. However, in 0.8 μm filtered water (Fig. 2.4.2 C) there was a decrease in total, viable and culturable numbers, mainly from four to eight dpi, until about 10^4 cells/ml and 10^3 CFU/ml respectively, and then stabilization around one log unit lower for the following three weeks. In untreated river water (Fig. 2.4.2 D), a reduction to around 10^5-10^4 cells and CFU/ml from two to four dpi was observed, which slowly continued, reaching about 10^4 cells/ml and 10^3 CFU/ml by eight dpi and then stabilizing until the end of the experiment. Culturable indigenous bacteria counts in 0.8 μm filtered and untreated

river water microcosms stabilized at about 10^4 CFU/ml in the first days of the experiment.

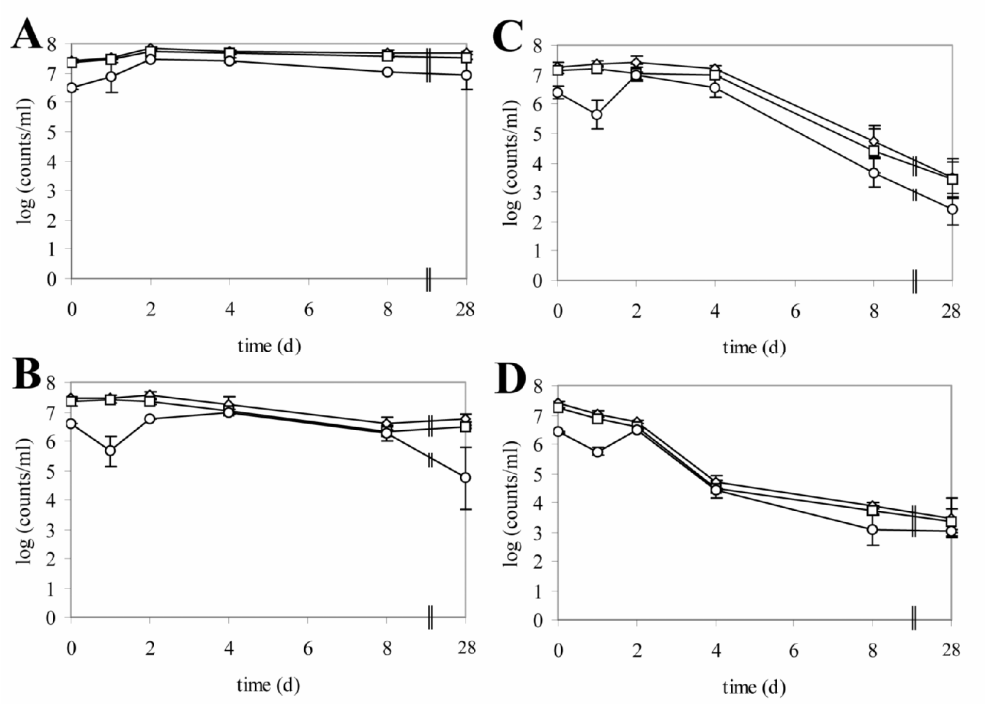


Figure 2.4.2. Dynamics of a selected phage-resistant variant of *R. solanacearum* ph II strain IVIA-1602.1: total (♦), viable (□) and culturable (○) cell counts on SMSA plates at 14ºC in river water microcosms. (A) sterile control water, (B) 0.2 μm filtered water, (C) 0.8 μm filtered water and (D) untreated water. Points are the mean of two separate assays in duplicate and error bars indicate variation as standard deviation for each point.

2.4.3.5 Interaction of *R. solanacearum* strain IVIA-1602.1 and a purified river water phage

The time-course of the interaction between *R. solanacearum* and one randomly selected phage was monitored at 24ºC and 14ºC in sterile river water for 24 h and 48 h respectively, although only data concerning the time of lysis needed to reach a minimum bacterial population are shown in Fig. 2.4.3. At 24ºC, for initial phage concentrations of $2x10^2$, $2x10^3$, $7x10^3$ and $2x10^4$ PFU/ml, bacterial populations decreased from 10^6 CFU/ml to values

ranging from 10^4 to 10^1 CFU/ml within 12 h (Fig. 2.4.3 A, B, C and D). Subsequent bacterial increases were observed due to phage-resistant variants (Fig. 2.4.3 A and D). With respect to phage populations, they either kept their levels or increased up to about 10^4-10^5 PFU/ml (Fig. 2.4.3 A, B, C and D).

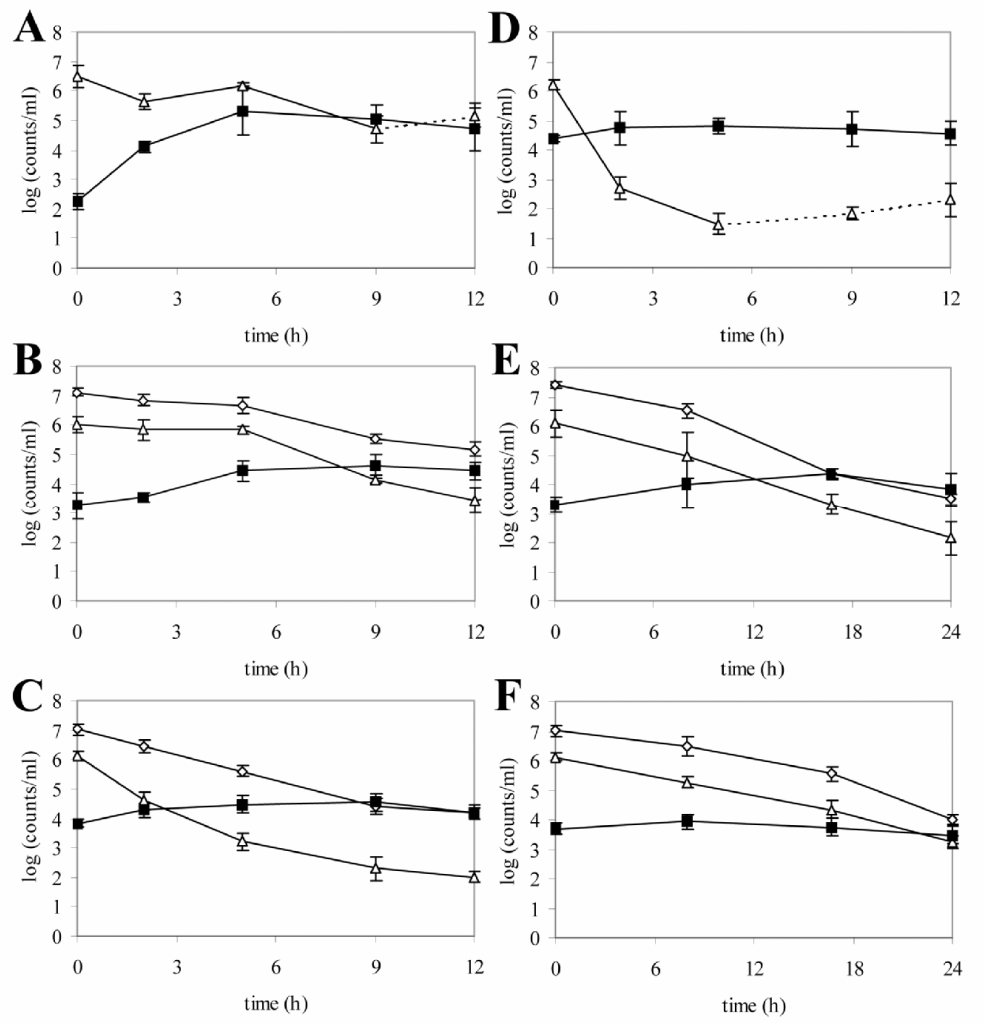


Figure 2.4.3. Time-course of the interaction between *R. solanacearum* ph II strain IVIA-1602.1 and a selected river water phage co-inoculated at different ratios in sterile natural water at 24ºC (A, B, C, D) and at 14ºC (E, F). Total *R. solanacearum* cell counts (♦) are shown only in B, C, E and F. Culturable *R. solanacearum* cells (▲) and plaque counts of the phage (■) were both on YPGA. Bacterium (CFU/ml): phage (PFU/ml) ratios were: (A) 10^6: $2x10^2$, (B, E) 10^6: $2x10^3$, (C, F) 10^6: $7x10^3$ and (D)

10^6: $2x10^4$. Broken lines (- - -) refer to a phage resistant variant of the inoculated strain of *R. solanacearum*. Points are the mean of two separate assays in duplicate and error bars indicate variation as standard deviation for each point.

At 14ºC, the pathogen populations decreased to around 10^3 CFU/ml within 24 h at both phage concentrations (Fig. 2.4.3 E and F), followed by stabilization at this level to the end of the experiments. Phage populations increased from 10^3 to about 10^4 PFU/ml (Fig. 2.4.3 E and F). Phage-resistant variants of the inoculated strain were also observed in some of the assays. In control sterile river water, *R. solanacearum* and phage populations remained at levels similar to the initial ones (data not shown).

Lysis of *R. solanacearum* cells by phages was more clearly appreciable after comparison between total and culturable bacterial cell counts, performed during the co-culture experiments for bacterium:phage ratios of 10^6 CFU/ml: $2x10^3$ PFU/ml and 10^6 CFU/ml: $7x10^3$ PFU/ml at both temperatures (Fig. 2.4.3 B, C, E and F). At 24ºC, *R. solanacearum* total counts decreased from the initial value of 10^7 cells/ml until 10^5-10^4 cells/ml in about 12 h, with culturability remaining two-three log units lower (Fig. 2.4.3 B and C). At 14ºC, *R. solanacearum* total counts decreased from 10^7 cells/ml to around 10^4-10^3 cells/ml by 24 h, with culturability remaining about one log unit lower (Fig. 2.4.3 E and F).

2.4.4 Discussion

This study details, for the first time, the influence of different fractions of native microbiota on the survival of *R. solanacearum* ph II in river water microcosms, trying to mimic conditions of water pollution by this pathogen as suggested to have happened in several watercourses (López & Biosca, 2005).

The number of indigenous protozoa and culturable bacteria in the river water samples was similar to others reported in freshwater systems (Caruso *et al.*, 2005; Servais *et al.*, 1995; van Elsas *et al.*, 2001). Likewise, population levels of culturable *R. solanacearum* ph II in these waters were in accordance with previous reports at water temperatures above 14°C

(Caruso *et al.*, 2005). Estimations of the relative abundance of *R. solanacearum* lytic phages in the river water were always higher than those of host cells, similarly to other studies in environmental waters (Weinbauer, 2004). The fact that lytic phages were isolated from the river water samples from which the bacterium was recovered suggests an association between them. Interestingly, positive phage detection at 29ºC always became negative when tested at 35ºC using the same water samples. This would explain previous results showing a more efficient recovery of *R. solanacearum* from samples from the same river by enrichment at 35ºC than at 29ºC (Caruso *et al.*, 2005), not only because the pathogen is able to grow at 35ºC over most of the native water bacteria, but also due to phage inactivation at this temperature.

With respect to the survival of *R. solanacearum* in control microcosms of sterilized river water, total, viable and culturable populations of the inoculated Spanish strain remained largely constant at inoculation levels at 24ºC and 14ºC throughout the experiments. The survival of this pathogen under the natural nutrient limitation conditions of aquatic ecosystems was thus confirmed since, according to previous studies (Kelman, 1956; van Elsas *et al.*, 2001; van Overbeek *et al.*, 2004), both temperatures favour its persistence. In contrast, *R. solanacearum* counts in the microcosms with water biota declined in total, viable and culturable cells within the first days, showing that a great proportion of the inoculated population was affected by native microbiota with lytic and/or predatory activity. A negative effect of biotic factors on *R. solanacearum* was previously reported in Dutch water microcosms but, after studying only the culturability, the authors did not analyse the nature of the agents involved (van Elsas *et al.*, 2001).

At 24ºC, decreases in microscopic counts observed within the first days were progressively more noticeable as more biotic fractions were present in the non-sterile microcosms, and for culturable counts declines were comparably more pronounced. Lytic activity by phages was likely to be involved, since all the non-sterile microcosms contained the river water viral fraction. Predation by other water microorganisms also contributed to the decline, when present in the non-sterile microcosms. The differences

between microscopic and CFU counts, especially in those microcosms with 0.2 μm filtered water, could be due to the physiological state of the host cells: when they are under nutrient-depleted conditions, as in river water microcosms, phages may be in an intracellular non-replicative state for an extended period and/or have a reduced replication rate (Morita, 1997; Weinbauer, 2004). On the contrary, for CFU counts, lysis occurred for the 72 h of incubation, with host cells actively growing on nutritive media. In the microcosms keeping only the viral fraction, the decline in culturability was followed a few days later by a slight increase due to the appearance of small and less fluid colonies with irregular edges. Such colonies were identified as *R. solanacearum* phage-resistant variants and were also observed in the experiments performed with the Dutch strain. Their existence might be hindering the observation of higher decreases in the populations of the inoculated strain by phage activity in 0.2 μm filtered water, when compared to 0.8 μm filtered and untreated water microcosms, from which these variants were not recovered.

The resistant variants would have appeared in the microcosms at 24ºC within the first 48 h, but they would have remained undetected because of their low population levels. Afterwards, growth of the variants would have taken place at the expense of leakage and cellular debris from phage-sensitive lysed cells. This phenomenon, called cryptic growth (Postgate, 1976), might be considered as a survival strategy that bacteria would employ to cope with adverse conditions (Roszak *et al.*, 1984). These phage-resistant bacterial populations remained about two log units lower than those of the inoculated strain. This could be explained by the metabolic cost often involved in the acquisition of bacterial resistance to phage infection (Gill & Abedon, 2003), resulting in a population that is not as well-competitive as the non-resistant population (Bohannan & Lenski, 2000).

At 14ºC, in non-sterile microcosms there were slighter declines in *R. solanacearum* populations when compared to those at 24ºC, which implied that biotic interactions were slower at a lower temperature. Similarly to what happened at 24ºC, in 0.2 μm filtered water colonies of the phage-

resistant variant appeared on plates, but simultaneously with remaining small populations of the inoculated strain. Such phage-sensitive populations were also observed on the selective medium from microcosms with 0.8 μm filtered water and with untreated river water. They were not affected by viral lysis probably due to their low numbers, which may be below a threshold for phage infection, as suggested in other models (Weinbauer, 2004; Wiggins & Alexander, 1985). This would result in an ecological balance between prey (strain IVIA-1602.1) and predator (bacteriophage) achieved within time in the river water microcosms, as observed in other freshwater systems (Weinbauer, 2004; Wiggins & Alexander, 1985). In the non-sterile river water microcosms, the temperature of 14ºC was more favourable for the survival of the pathogen than 24ºC.

Trends in the dynamics of *R. solanacearum* populations in the different river water microcosms were similar for the Dutch strain, suggesting that the biotic effects influencing the pathogen persistence were not related only to one strain.

The influence of non-specific protozoan grazing and/or bacterial activity on *R. solanacearum* survival could be observed more precisely in the experiment in which the river water microcosms were inoculated with a selected phage-resistant variant that exhibited similar cellular size to that of the wild type. In untreated water, the decline in the pathogen populations after two to four days pointed out the protozoa grazing *R. solanacearum* cells, released into the microcosms in larger numbers than native bacteria. A similar period for protozoa to reach sufficient density to effect a detectable removal of *E. coli* in natural water has been reported at a similar temperature (Guixa-Boixereu *et al.*, 1999). Afterwards, a slight decrease in population was observed in untreated water which was more noticeable in 0.8 μm filtered water microcosms, and would be due to predatory activity by remaining flexible small-sized protozoa able to pass the filters. No increase of the *R. solanacearum* populations occurred during the following three weeks of the experiment, with such populations remaining at densities lower than those of competitor native bacteria that grew faster than *R. solanacearum* on plates. Bacterial competition for the scarce water

nutrients was also observed in the experiments with the phage-sensitive strain, where the phage-resistant variant populations could increase only in the non-sterile microcosms without native bacteria. This is in agreement with other studies reporting the influence of indigenous protozoa and bacteria on other bacterial species introduced in freshwater (Arana *et al.*, 2003; González *et al.*, 1992; Guerrero *et al.*, 1986; Gurijala & Alexander, 1990).

The time-course of the interaction between *R. solanacearum* and a selected river water phage in sterile river water confirmed the lysis of bacterial cells at different phage concentrations, and at 24ºC and 14ºC. In spite of the fact that bacterial lysis by phages is usually proved by culturable data, in this work it has been furtherly demonstrated by microscopic counts of total bacterial cells. Thus, at both temperatures, in the absence of native water protozoa and bacteria, lysis occurred as in non-sterile river water microcosms, as evidenced by declines in both total and culturable cell numbers, from millions to several thousands or hundreds of *R. solanacearum* cells. Differences between total and culturable counts at 24ºC might be due to starvation conditions as already discussed. At 14ºC, smaller differences were recorded probably because of a lessened effect of starvation when the host has lower metabolic activity, and the lysis was delayed in time.

In summary, this study has revealed that *R. solanacearum* survival was less favoured in non-sterile than in sterile river water microcosms and that biotic factors influenced this persistence in freshwater. After an increase in *R. solanacearum* populations in environmental watercourses (due to either occasional spilling or dumping of polluted waste, multiplication and leaching from infected roots of *Solanum dulcamara* or others), lytic phages would reduce the densities of the bacterium and also protozoa and bacteria may have an influence on *R. solanacearum* abundance. All this complex interactions would be affected by water temperature. Evidence presented in this work may broaden our knowledge on the epidemiology of the bacterial wilt pathogen in environmental waters, which might improve the strategies for the management of the disease.

Acknowledgements

B. Álvarez thanks the Instituto Valenciano de Investigaciones Agrarias for a predoctoral grant. The authors wish to thank J.L. Palomo and the Consejería de Agricultura de Castilla-León for collecting the river water samples, J.D. van Elsas for strain IPO-1609, P. Caruso, E. Bertolini, J. Penyalver, M. Gil and V. Herrera for technical assistance, E. Carbonell and J. Pérez Panadés for statistical analysis and F. Barraclough for English revision of the manuscript.

This work has been funded by projects FAIR 5-CT97-3632 and QLK 3-CT-2000-01598 of the European Union and FD 1997-2279 of the Ministerio de Educación y Ciencia of Spain.

PART 3
GENERAL DISCUSSION

R. solanacearum is a bacterial species extensively investigated since late 19th century, after its isolation and confirmation as phytopathogenic (Smith, 1896). Notwithstanding, most of the research work has been done with race 1 strains such as the type strain of the species, belonging to race 1 bv 1 and isolated from tomato in the USA (Bergey's, 2004). The reason for this race being more frequently investigated is that it has long been present in crop fields in the USA causing epidemics mainly to tobacco, tomato and potato plants and has also been repeatedly reported causing disease in a wide range of hosts worldwide (Elphinstone, 2005). Likewise, research in Europe carried out mainly in France has mostly been conducted with a strain belonging to race 1 bv 3, originally isolated from tomato in French Guyana (South America).

Consequently, recent introduction (occurred from the last decades of the 20th century) of race 3 bv 2 strains of the species complex into several EU countries, among them Spain, and the USA, where they are considered as quarantine pathogens (Anonymous, 2000; Lambert, 2002), has raised a great many questions concerning aspects of the biology of the bacterium and the epidemiology of the disease in these temperate zones, whose answer would contribute to assess the potential risk of a successful dissemination and establishment of the pathogen to these new areas.

Accordingly, this work was undertaken to try to respond to some of these questions, particularly by making a contribution to the knowledge of the behaviour, the ability for survival and the disease inducing capacity of European *R. solanacearum* race 3 bv 2 strains confronted with a number of natural environments other than those of the best known susceptible hosts (Smith, 1920; Kelman, 1953; Vasse *et al.*, 1995, 2005).

On that purpose, the research was aimed to cover a broad spectrum of environments mimicking those the pathogen might have to cope with in European cultivated fields, river banks with semi-aquatic weeds and/or surface run-off waters. In this respect, up to 20 plant species belonging to different botanical families and most of them of economic interest in European agriculture were selected, as well as the weed *S. dulcamara* present in European river banks and related to *R. solanacearum* detections in

EU countries (Elphinstone *et al.*, 1998; Palomo *et al.*, 2002). Also a range of water samples was collected in Spain in river locations where the pathogen had previously been detected, and the influence of abiotic and biotic factors namely oligotrophy, temperature and indigenous water microbiota was addressed in them. Race 3 bv 2 *R. solanacearum* strains used for the research were IPO 1609 and IVIA 1602.1, isolated from diseased potatoes in cold (The Netherlands) and warm (Spain) areas respectively, in the first years of detection of the pathogen in the EU.

Knowledge of the behaviour of *R. solanacearum* race 3 bv 2 in the large set of plant species abovementioned can be useful to manage cropping systems in the fields where the pathogen has been detected or constitutes a real threat. The methodology used in the work based on histological methods and bacterial isolation following a challenged inoculation with a β-glucuronidase-expressing strain IPO 1609 has proved easy to use. Histological localization *in planta* performed by examination of a great many plant segments embedded into a GUS reactive agar has made it possible to specifically describe the localization of the derivative on surface, in cortex and/or in xylem of plant roots and stems, and eventually to classify the plant species as susceptible or tolerant hosts, or as non-hosts for the pathogen.

The classification scheme set up in this work defines susceptible and tolerant hosts as those invaded by high densities of the pathogen in xylem elements at the root level, although there is a remarkable difference at the stem level: in susceptible plants invasion of xylem at the middle part of the stem is heavy, while in tolerant plants it becomes strongly limited. Non-hosts are defined as those in which no xylem invasion is observed, though some external contamination of the rhizoplane and rare cortical infection pockets may occur. This carefully-conceived classification system provides with diverse advantageous characteristics. On the one hand, it offers the possibility to differentiate between true and putative host plants, and therefore to identify new hosts that should not be cultivated in areas where there is a risk of bacterial wilt outbreak. On the other hand, it allows to easily identifying suitable non-host candidates to be used as rotation crops in *R. solanacearum*-threatened plots. All this knowledge may contribute to

improve the development of integrated systems in bacterial wilt management programmes.

Thus, according to the classification, the plant species and cultivars tested in this work have been included into any of the three categories.

Susceptible hosts have been forage cabbage cv. Proteor, cabbage cv. Quintal d'Alsace and curly endive cv. Rida Doble de Verano, since the β-glucuronidase-expressing derivative was present in cortex and xylem of roots and lower parts of stems and was isolated from middle part of stems in all plants. They are to be avoided in a crop rotation scheme.

Likewise, tolerant hosts have been cabbage cv. Virtudes, rutabaga cvs. Champion and Whilemsburger and kidney bean cv. Coco Blanc, since the derivative was observed in cortex and/or xylem of roots and/or lower parts of stems in only a few individuals and isolations from the middle part of stems were mostly negative. They are not recommended as rotation crops.

Finally, non-hosts have been barley cv. Cork, black radish cv. Gros Long d'Hiver, fiber flax cv. Hermès, field bean cv. Maya, field pea cv. Solara, horseradish, maize cv. Symphony and zucchini cv. Virginia, where the derivative was only observed in some root cortex areas for some of the plant species but never in inner root or stem tissues, and also alfalfa cvs. Gea R1 and Symphonie, carrot cvs. Nantaise and Tempo, celery cv. Istar, colocynth, and fennel cv. Doux de Florence, where the derivative was never observed in inner root or stem tissues, although it was detected on the roots in some of the plant species. They could be selected as candidates for crop rotation but, as some of them might somehow constitute a protecting shelter for *R. solanacearum*, tests under field conditions would be required, at least in humid areas, to effectively know whether some of these candidates act as a reservoir for the pathogen.

Regarding the semi-aquatic bittersweet nightshade, the localization of *R. solanacearum* in the tissues of this weed has been visualized for the first time and it has been classified as a tolerant host. Therefore, the general belief that this weed is a symptomless carrier of the pathogen (Elphinstone, 2005) has been eventually confirmed.

Susceptible hosts of *R. solanacearum* as tomato plants cv. Roma and potato (plants and tubers) cvs. Désirée and Spunta have been the valid controls of the methodology for the classification scheme. Histological localization of the pathogen in them has been in agreement with that described elsewhere (Smith, 1920; Kelman, 1953; Vasse *et al.*, 1995). In tubers, it has been definitively confirmed that eyes and stolon heel ends are the main entrance sites of the pathogen, since it had been only suggested (Kelman, 1953) up until now.

With respect to the methodology, experimental procedures performed under axenic and non-axenic conditions have yielded *R. solanacearum* localizations in the same zones of the plant tissues, although some plant species have been more frequently and intensely colonized under axenic conditions, characterized by extremely high humidity. Thus, axenic conditions would rather be mimicking conditions encountered in temporary swampy areas, whereas non-axenic conditions would correlate better with conditions of areas in the absence of temporary excess of water.

This work has revealed new insights into the colonization process of hosts and non-hosts of *R. solanacearum*. Bacterial colonization has considerably varied, even among species within a plant family, and appears to depend on the plant species and cultivar tested and presumably also on the strain biovar and aggressiveness. As a consequence the concept of host range of the *R. solanacearum* species complex should be adapted to the native crops and strains of each particular geographical region.

The research carried out can be considered as very useful, since data on the response of commonly-grown crops to the pathogen is vital in designing the rotation strategies to be used after outbreaks of the disease. Furthermore, an incomplete and somewhat haphazard set of individual reports has been replaced by an approach that allows comparisons across species and can be extended to any crop or plant species of interest. The methodology is intended to contribute to the bacterial wilt disease prevention. It lays the groundwork for field testing and eventual practical recommendations for effective pathogen management by crop rotation.

Due to the fact that surface run off waters can be a means of dissemination of *R. solanacearum* race 3 bv 2, a great many river water

samples from diverse locations of the river Tormes in Spain were used to specifically ascertain the influence that abiotic factors like oligotrophy and temperature, and biotic factors like indigenous microbiota have on the ability for survival and disease inducing capacity of the pathogen.

Oligotrophy and temperature are major environmental abiotic factors that are fundamental for bacterial persistence and growth in aquatic ecosystems. Although there are some previous interesting works concerning a number of abiotic factors affecting *R. solanacearum*, they did not concern with survival and pathogenicity but only with culturability of the pathogen in water (Kelman, 1956; van Elsas *et al.*, 2001).

With respect to oligotrophy, the survival ability of the pathogen in environmental water microcosms was assessed over four years without addition of any nutrients. The period has been the longest ever reported for persistence of *R. solanacearum* in aquatic non-host environments. The study has been performed in river water microcosms in the absence of stresses other than oligotrophy, in order to monitor the survival of the bacterium as influenced uniquely by this factor, considered to be the most common environmental stress affecting persistence of bacteria in aquatic habitats (Morita, 1997). Thus, indigenous water microbiota was removed by water filtration and sterilization, and the pathogen was exposed during the four years to the compromised temperature of 24°C, an environmental value near to the optimum of the bacterium.

This work has revealed an impressive potential for subsistence and maintenance of pathogenicity of *R. solanacearum* race 3 bv 2 in energy-deficient systems. The bacterium has managed to survive for four years in oligotrophic river water microcosms, indicating that it is an efficient scavenger of scarce nutrients, and has further proved to successfully be able to retain both infective and disease-inducing capacities in the host during this whole starvation period.

Furthermore, a number of survival strategies have been firstly described for the pathogen favouring its persistence under these prolonged stressful conditions of scarcity of nutrients in environmental waters. One of the survival mechanisms has been a starvation-survival response, that is, a

physiological state wherein bacterial cells undergo significant decreases in metabolism permitting the maintenance of the starved populations for long periods of time in a non-growing but culturable state, since there is no sufficient energy for normal growth and reproduction (Morita, 1997).

Another mechanism has been the entrance of proportions of viable *R. solanacearum* populations into a VBNC state. This work constitutes the first report on the induction of the VBNC state in *R. solanacearum* caused by extended nutrient deprivation conditions, which has been demonstrated by employing two different methods to monitor and verify viability. VBNC significance lies in the fact that cells in this physiological state are more difficult to be detected from the environment by cultivation-based methods [in the EU, required by legislation (Anonymous, 2006) to determine the presence of the pathogen], together with the fact that their potential pathogenic capacity remains unclear. They presently represent a challenge to be overcome in bacterial wilt disease management, and further research should be carried out to specifically address the role of the VBNC state in the life cycle of *R. solanacearum* in natural settings.

The occurrence of cell divisions in the starved *R. solanacearum* populations has also been a strategy facilitating survival in the river water microcosms. The increase in cell numbers would provide with a selective advantage to the stressed populations in terms of raising the probability of one cell successfully disseminating towards new ecological niches to be colonized. The energy for these divisions to take place would have been probably obtained by cryptic growth from degradation of non-viable cells in the microcosms. Moreover, marked morphological changes have been observed in the starved *R. solanacearum* cells, since there has been a progressive conversion of the typical bacillary shape into smaller and rounded coccoids. Reduction in cell volume and acquisition of cell round shape are survival mechanisms adopted to simultaneously increase the probability of water nutrient capture and decrease the cell energetic requirements (Roszak & Colwell, 1987; Morita, 1997). Together with an increase in the population numbers, these smaller and rounded cells would have appeared in the water microcosms by the occurrence of reductive divisions, a survival strategy which allows the small cocci to successfully

colonize more intricate places searching for food and protection (Morita, 1997; Byrd, 2000).

The rise in the *R. solanacearum* populations is also likely to have been caused by filamentation and budding phenomena, observed later than reductive divisions in the microcosms. A proportion of *R. solanacearum* race 3 bv 2 cells has been observed to filament under conditions of extended nutrient limitation in the river water microcosms. Some of the filaments have been noticed to have cell wall evaginations or buds. It has been hypothesized that the elongated, budded forms are valid transients in the process of coccoid formation, in which the buds expand due to the migration of cell components from the elongated suspensor cell, developing coccoid forms that eventually become detached (Thomas *et al.*, 1999). To our knowledge, this has been the first report of filamentation and budding phenomena occurring in a plant pathogenic bacterium.

Another mechanism for survival that has been noticed to take place in the river water microcosms in prolonged harsh conditions is bacterial aggregation. Long starved *R. solanacearum* coccoid forms have displayed a frequent tendency to group in aggregates, where the cells would be more protected from toxic substances and predators (Blat & Eisenbach, 1995).

The research has furnished with data proving that *R. solanacearum* race 3 bv 2 has kept a disease-inducing capacity in susceptible host plants during the four years subjected to nutrient limitation conditions in the river water microcosms. This is the longest period ever reported for this pathogenic agent being effectively able to wilt and destroy the host. In fact, a similar disease incidence has been found with stem-inoculated *R. solanacearum* either starved up to four years or freshly grown, which points out to a great capacity of this pathogen to retain virulence. Furthermore, long-starved *R. solanacearum* has also proved to succeed in wilting the host by watering, suggesting that a real risk of water-borne transmission of this pathogen after extended periods of starvation in environmental waters should not be ruled out, since the number of litres used for irrigation may represent a massive density of cells reaching the host roots in spite of

relatively low *R. solanacearum* concentrations in water (Lopez & Biosca, 2005).

On the other hand, due to the fact that culturable cells persisted so long, the *R. solanacearum* VBNC cells from the river water microcosms could not be shown to cause infection in the host and therefore resuscitation of these VBNC cells could not be proved to have occurred *in planta*, contrarily to what reported elsewhere (Grey & Steck, 2001). Notwithstanding, these *R. solanacearum* VBNC populations have been hypothesized to be composed of a co-existence of cells viable enough to be resuscitated in favourable restored conditions, and of cells unable to resume active growth, which would be just dying-off, in accordance to the model by Shleeva *et al.* (2002). Non-viable cells have been also clearly observed. However, towards the end of the four years of starvation, the viability criterion based on metabolic response has appeared to be more stringent than that based on membrane integrity.

Faced with the evidence, *R. solanacearum* race 3 bv 2 has displayed an exceptional ability for survival, culturability and pathogenicity under starvation during periods of time as long as four years. It has been outstanding the fact that the bacterium has borne commonly prevalent oligotrophic characteristics of aquatic habitats by exhibiting a number of valid strategies within time. This research can contribute in some manner to improve the knowledge of *R. solanacearum* epidemiology, especially with regard to develop innovative preventive measures to avoid the dissemination of the pathogen and the propagation of the bacterial wilt disease in the environment.

To assess the effect of temperature on the survival and pathogenicity of *R. solanacearum* race 3 bv 2 in freshwaters, strains introduced to temperate areas and isolated from either cold or warm habitats have been subjected to three different environmental temperatures [cold (4°C), temperate (14°C) and warm (24°C)] in water microcosms at two different levels of oligotrophy [nutrient-deprived (distilled water) and nutrient-limited (natural river water)].

The monitoring of the inoculated populations has permitted to describe in this work distinct survival strategies by *R. solanacearum* race 3

bv 2 undergoing simultaneous stresses of temperature and oligotrophy. The pathogen has remained adaptable, capable of responding to these conditions more approaching those of natural settings, and has managed to preserve life and disease-inducing capacity on host plants.

Under low temperature conditions, viable *R. solanacearum* populations starving in the microcosms, originally coming from either a cold or warm climate, have been similarly and progressively induced into the VBNC state, which had been also reported for *R. solanacearum* in pure water at 4°C (van Elsas *et al.*, 2001; van Overbeek *et al.*, 2004). This physiological response, known to be activated in adverse external environments, has appeared to be an adaptation to coldness. Thus, it has been confirmed that a steady exposure at the temperature of 4°C is stressful for *R. solanacearum* race 3 bv 2 introduced to temperate areas even if isolated from cold habitats, suggesting a lack of natural cold-adaptation capacity. Furthermore, time for cold-induction of VBNC *R. solanacearum* in water microcosms has proved to be dependent on water nutrient contents, since nutrient deprivation conditions have markedly shortened the VBNC induction period in comparison to conditions of nutrient limitation. The differences found between the two levels of oligotrophy have corroborated the relevance of selecting conditions bearing a strong resemblance to those commonly occurring in natural settings. It can be hypothesized that the more limiting for *R. solanacearum* growth water nutrient concentrations are, the more efficient they may act as an additional stress contributing to the cold-induction of the VBNC state. Thus, natural nutrient availability of environmental waters may be protecting the pathogen from low-temperature stress, favouring its survival without affecting its pathogenicity. On the other hand, a practical conclusion of the potential existence of VBNC cells in watercourses, together with the fact that the temperature interval applied by EU legislation to transport suspected water samples is from 4°C to 10°C (Anonymous, 2006), have led to propose in this work the recommendation of keeping the samples at temperatures around 10°C rather than at 4°C. In fact, since isolation of the pathogen is an

undisputed requisite for a positive detection (Anonymous, 2006) conditions that lead to the VBNC state must be avoided.

Under temperate and warm temperature conditions, the strategies exhibited by *R. solanacearum* populations to withstand nutrient scarcity within time have been starvation-survival responses. At both temperatures the starved populations have remained similar in terms of population numbers, with a majority of cells readily culturable and pathogenic on the host. Likewise, the presence of trace organic matter and some dissolved salts has appeared to favour culturability of the pathogen in environmental water microcosms at both temperatures.

Morphological changes in starved *R. solanacearum* cells have also been observed in the water microcosms, but only with significance in those subjected to temperate and warm temperatures. Under low temperature conditions, a transition from the typical bacillary cells to coccoids has hardly taken place, most likely due to a quick stressful effect of coldness on cell metabolism. On the contrary, under temperate and warm conditions, proportions of *R. solanacearum* coccoid forms have appeared because of cell starvation in the water microcosms. Notwithstanding, this adaptation to oligotrophy aimed to optimize the ratio between nutrients captured and energy required in starving cells has been more strongly stimulated under warm conditions. This has suggested a role of temperature in contributing to the stress caused by oligotrophy when acting simultaneously at temperature values allowing bacterial multiplication. According to that, it has been hypothesized that higher temperatures might exert higher pressures on the cell by accelerating metabolism rate and therefore increasing cell nutrient requirement. As a consequence, it has been pointed out that the stress of oligotrophy on the *R. solanacearum* race 3 bv 2 introduced to temperate areas might be less intense at temperatures around 14°C than at values nearer to the optimum of the pathogen, such as 24°C, then favouring survival at 14°C.

An outstanding accomplishment of this research has been the knowledge provided on the interaction between two environmental factors acting simultaneously on the bacterial cell. Thus, it has been inferred from this interaction that one of the factors would be acting as a main stress

whilst the other would rather be contributing to it as a secondary stress, with these roles being reversed depending on the conditions of the factors. In this respect, temperature has been the main stress in cold conditions, where the lowest level of oligotrophy, nutrient deprivation, has accelerated the entrance in the VBNC state in comparison to nutrient limitation conditions. Likewise, oligotrophy has been the main stress in temperate and warm conditions, with similar effects on the bacterial populations at both levels of oligotrophy, and temperature has differently contributed by stimulating the transformation of cells into coccoids at higher values (at 24°C). Thus, *R. solanacearum* race 3 bv 2 introduced to temperate areas has survived and remained pathogenic in all condition, and has appeared to be better temperate-adapted than cold-adapted, regardless of being isolated from either cold or warm areas. Altogether, this work has furnished with information on *R. solanacearum* race 3 bv 2 behaviour when subjected to a combined effect of environmental stresses that can be useful to improve measures to prevent the dissemination of the pathogen in European watersystems.

With respect to the effect of biotic factors, this work has provided with the first convincingly evidence of the influence of different fractions of river water microbiota on the survival of *R. solanacearum*. Differential filtration of river water where the pathogen had previously been detected was performed to prepare river water microcosms containing different microbiota fractions, that is, indigenous protozoa, bacteria and bacteriophages, which were enumerated. The microcosms were inoculated with the pathogen and the populations carefully monitored in the microcosms at 24°C and 14°C [in the range of *R. solanacearum* detection in the river (Caruso *et al.*, 2005)] in order to establish their progression. The interest of the work lies in the fact that such microcosms would be mimicking what would be happening in the river water when *R. solanacearum* concentrations might increase, for instance in case of warm temperatures, nutrient input, presence of *S. dulcamara*, that may hold large populations of the pathogen, and occasional spilling or dumping of polluted waste. Furthermore, from an ecological point of view, inoculation

broke an established balance among populations and the possibility for them to regain a balance anew might also be assessed.

The research has consistently revealed that persistence of *R. solanacearum* race 3 bv 2 in freshwater has been much less favoured in microcosms with microbiota (protozoa, bacteria and/or bacteriophages) having lytic, predatory or competitive activity than in sterile controls, since the inoculated populations, composed of total, viable and culturable *R. solanacearum* cells, clearly declined within the first days only in the non-sterile microcosms at both temperatures, 24°C and 14°C.

The effect has been more remarkable at 24°C, since the inoculated populations decreased their numbers in great proportions as soon as 24-48 hours after inoculation, and in a more noticeably manner as more biotic fractions were present in the microcosms. Due to the fact that lytic phages for *R. solanacearum* had been detected in the river water and were present in all these non-sterile microcosms, they have been considered as the main responsible agents for the impact on *R. solanacearum* survival in the river water, although there have been a simultaneous contribution of other water microorganisms when present.

Regarding these phages, to our knowledge this is the first time that bacteriophages able to lysis *R. solanacearum* have been detected and isolated from watersystems. Their detection has been positive at 29°C but negative at 35°C, although their concentrations in the river water have always been higher than those of the host cells. Phage inactivation at 35°C demonstrated in this work has been proposed as one of the reasons explaining the fact that *R. solanacearum* was more efficiently recovered from environmental water samples by enrichment at 35°C than at 29°C, as it had been reported (Caruso *et al.*, 2005). The mechanism behind inactivation at 35°C appears to be a potential matter for further research. Interestingly, the isolation of these phages have been possible only from the river water samples from which the bacterium had also been recovered, which have suggested an association between the pathogen and its river water phages that remains undetermined. A complete biological and molecular characterization of these river water phages certainly constitutes the basis for future research aimed to elucidate the ecological role of these bacteriophages in the life

cycle of *R. solanacearum* and the possibility of successfully exploiting them in bacterial wilt management programmes.

The continuous pressure of the predators (the bacteriophages) onto their prey (the inoculated *R. solanacearum*) led to the appearance of phage-resistant *R. solanacearum* variants in the microcosms containing only the viral fraction of the river water. It should be noted the way in which the pathogen has quickly evolved to become resistant to the indigenous phages, since the resistant populations reached detectable levels just a few days after the inoculation. Notwithstanding, it is worthy of remark that this adaptation was of little benefit when other river microorganisms were present in the microcosms, which better corresponds with more realistic conditions in natural ecosystems. These phage-resistant populations being hindered from appearing is most probably due to the effect of bacterial competition for nutrients and space taking place in the microcosms by active indigenous bacterial populations already established in them. *R. solanacearum* resistance to phages might be mainly due to either lysogeny or LPS defectiveness. Both of them frequently lead to alteration of host cell phenotype, which is in agreement with the smaller and less fluid colonies with irregular edges of the phage-resistant variants observed from the microcosms. Accordingly, further research would be needed to characterize this phage resistance by *R. solanacearum* and determine the conditions for it to develop.

The effect of the complex interactions among microorganisms on *R. solanacearum* race 3 bv 2 survival at the lower temperature of 14°C has proved to be slower and less intense than that at 24°C, since the inoculated phage-sensitive populations decreased in a lesser extent within time, therefore remaining in low numbers in the non-sterile microcosms. They have been observed simultaneously with phage-resistant populations that have appeared, at least in a detectable level, in the microcosms containing only the viral fraction and not in those with other microorganisms, like at 24°C. Interestingly, a balance between predator and prey appears to have been established which, hypothetically, would explain the occurrence of *R. solanacearum* in the river water despite the presence of lytic phages. The

conditions for this balance to take place within time might be low phage-sensitive population numbers, remaining below a threshold for phage infection.

At both temperatures, 24°C and 14°C, the lytic activity of a selected river water phage on *R. solanacearum* cells at different phage concentrations in sterile river water has been further confirmed in the absence of indigenous water protozoa and bacteria, and additionally quantified by decreases in total and culturable *R. solanacearum* population numbers, from millions of cells to several thousands or hundreds of them. Thus, the impact of the lysis by native river water bacteriophages on the pathogen, initially observed in non-sterile water in conditions more approaching those of natural settings, has been also demonstrated in a more controlled system consisting of sterile water, inoculated pathogen and inoculated bacteriophage.

The effect of river water protozoa and other bacteria on *R. solanacearum* race 3 bv 2 survival has also been observed in this work, apart from that of bacteriophages. As this effect would have been masked by the stronger activity of phages in the microcosms with the phage-sensitive strain, a selected phage-resistant variant has been inoculated to assess it. The fact that protozoan grazing is a non-specific process depending on bacterial cell size and bacterial population numbers made this choice suitable, since cell sizes of wild type and phage-resistant variant were similar and the variant was released into the microcosms in larger numbers than indigenous water bacteria, as it had been performed with the wild type in previous experiments. Thus, a detectable removal of *R. solanacearum* cells (total, viable and culturable) in these river water microcosms has been attributed to grazing by populations of diverse protozoan species, which have been microscopically visualized. Then, a few days after inoculation significant predation could be observed after protozoa had reached enough density in the microcosms. Thereafter no increase in *R. solanacearum* populations has been noticed, not even at the expense of leakage and cellular debris from dead protozoan and bacterial cells naturally occurring in the microcosms (cryptic growth), most likely due to competition events by the indigenous river water bacteria, whose population densities

remained higher than those of *R. solanacearum* and grew faster than the pathogen on plates. The existence of a balance between predator and prey in these microcosms is difficult to state due to the fact that different protozoan species are non-specifically and simultaneously predating different bacterial species, in contrast to the bacteriophages specifically lysing *R. solanacearum* cells.

As a whole, in non-sterile river water the temperature of 14°C has been more inductive for the persistence of the pathogen than 24°C, since the effect of the indigenous microbiota has proved to be slower and less intense. Likewise, as already mentioned, in sterile river water the stress of oligotrophy has been more moderate at 14°C than at 24°C. Therefore, it appears that *R. solanacearum* race 3 bv 2 survival in environmental waters can be more favoured at temperate temperatures as 14°C than at warm temperatures as 24°C, in spite of being less distant from the optimum of the bacterium. This observation, together with the presence of asymptomatic carriers and/or plant debris in the surroundings, may explain the long-term persistence of *R. solanacearum* race 3 bv 2 in aquatic habitats, which has been reported for periods as long as three years in environmental watersystems of temperate zones (Caruso *et al.*, 2005; Hong *et al.*, 2005). Thus, in common European environmental river banks with reservoir plants as *S. dulcamara*, *R. solanacearum* can shelter and multiply in them, and leaching of the cells into the river from the infected aquatic roots at temperatures above 14°C may be successfully counteracted by the effect of biotic factors. Protozoa, bacteria and bacteriophages would probably have a role in controlling the pathogen populations. Accordingly, the influence of biotic factors may explain the maintenance of *R. solanacearum* populations in low numbers in river systems free from hosts and reservoir plants. Water temperature and the oligotrophic conditions of the aquatic environments would be affecting all these complex interactions. This work has provided with novel and valuable information on the combined effect of biotic and abiotic factors influencing *R. solanacearum* race 3 bv 2 survival in freshwaters that will be useful to improve strategies (see section 1.3) for the

specific management of the bacterial wilt pathogen in natural environmental watercourses.

Throughout the research, trends in the dynamics of *R. solanacearum* race 3 bv 2 populations in the different water microcosms and their dynamics of adaptations have been similar for the two strains used, IPO 1609 and IVIA 1602.1, suggesting that the effects of the biotic (indigenous water microbiota) and abiotic (oligotrophy and temperature) factors on pathogen persistence, adaptations and pathogenicity have not been related only to one strain.

Prior to this work, knowledge of relevant aspects of the biology and behaviour of *R. solanacearum* race 3 bv 2 in a wide range of natural environments comprising susceptible and tolerant host plants, reservoir plants, non-host plants and aquatic habitats was somehow still scarce. It is true that at present there is some work remaining to be done, such as open field trials or *in situ* survival experiments. It would be also interesting to look into the interactions among indigenous water microorganisms and *R. solanacearum* in more detail. In spite of that, the findings of this research have certainly made significant contribution to the understanding of the ecology of *R. solanacearum* race 3 bv 2 and the epidemiology of the bacterial wilt disease, and have paved the way for further future work concerning the fields of bacterial adaptation, plant pathogenicity and disease prevention of this devastating pathogen.

PART 4
CONCLUSIONS

To conclude with the presentation of this research work, the originality and novelty of some results with respect to the control of the disease and the survival ability of the pathogen are emphasized in this section, as well as some new prospects that follow from these results.

Control of the disease by use of tolerant or non-host crops

1. A classification scheme for *R. solanacearum* based on histological localization *in planta* and bacterial isolation has been set up, which allows differentiating among susceptible hosts, tolerant hosts and non-hosts for the pathogen. This classification can be useful to establish a distinction between hosts and non-hosts, identify new hosts and reservoir plants, find candidates for crop rotation, and therefore to improve bacterial wilt management programmes.

2. According to the classification, for *R. solanacearum* ph II race 3 bv 2 the cultivars tested of the following plant species have been considered:

- susceptible hosts: forage cabbage and curly endive
- tolerant hosts: rutabaga and kidney bean
- either susceptible or tolerant hosts: cabbage
- non-hosts (reservoir plants under particular conditions): barley, black radish, fiber flax, field bean, field pea, horseradish, maize and zucchini
- non-hosts: alfalfa, carrot, celery, colocynth, and fennel

and therefore members of the same plant family, such as *Brassicaceae* and *Leguminosae*, have been classified into different categories; also members of the same plant species but distinct cultivar have been differently classified.

3. The histological localization of *R. solanacearum* ph II race 3 bv 2 *in planta* has definitively demonstrated that *S. dulcamara* is a tolerant host and so a symptomless carrier, and that eyes and stolon heel ends are the main entrance sites of the pathogen in tubers.

4. The concept of host range of the *R. solanacearum* species complex should be adapted to the local plant species and cultivars grown in a particular geographical region and to the specific strains colonizing these areas.

5. The methodology used with the plants, based mainly upon a rapid and simple binocular examination of hand-cut plant fragments embedded in a GUS reactive agar, has proved easy to use. Moreover, a great many plant segments of the same cultivar can be easily assessed, what may allow a solid statistical analysis and strengthen the status host *vs*. non-host of the plant species under study.

Survival abilities of the pathogen in different environmental conditions

6. Faced to abiotic and biotic factors in environmental water microcosms, *R. solanacearum* ph II race 3 bv 2 survival has proved to be less favoured by the effect of biotic factors like indigenous river water microbiota than by the effect of abiotic factors like oligotrophy and temperature.

7. *R. solanacearum* ph II race 3 bv 2 has proved great endurance when coping with nutrient scarcity. The pathogen has managed to survive over four years in oligotrophic environmental waters and has been able to retain disease-inducing capacity in the host during the whole period, revealing a high potential for subsistence in energy-deficient systems.

8. A number of strategies have been undertaken by the pathogen during the four years of nutrient limitation, namely starvation-survival responses and entrance into the VBNC state of part of the populations, increase in cell numbers, reduction in cell volume and acquisition of cell round shape, filamentation and budding phenomena, and cell aggregation.

9. Starved cells of *R. solanacearum* ph II race 3 bv 2 have kept pathogenicity and have been able of water-borne transmission of the disease over four

years, pointing out to the role of this means of dissemination in the persistence of bacterial wilt in the environment.

10. *R. solanacearum* ph II race 3 bv 2 has distinctly adjusted to simultaneous stressful conditions of temperature and nutrient limitation in water. In this respect, the strategies exhibited by the pathogen populations have enabled them to readily survive in oligotrophic environmental waters at different temperatures and keep their capacity to induce disease in susceptible hosts.

11. Steady exposure of *R. solanacearum* ph II race 3 bv 2 to 4°C has led to an induction of the populations into a VBNC state as an adaptation to coldness influenced by water nutrient contents. At 14°C and 24°C, faced to oligotrophy the pathogen has adopted starvation-survival responses and morphological changes, influenced by temperature. Thus, one of the factors appears to exert the main stress while the other would be contributing, with the roles being reversed depending on the conditions of the factors.

Survival abilities of the pathogen in the presence of native water microbiota

12. The activity of microorganisms present in river water has influenced *R. solanacearum* ph II race 3 bv 2 persistence. Each of the different fractions of the water microbiota has contributed to the significant decreases in the pathogen populations. Lytic activity by water bacteriophages has been the main responsible for the declines, although predation by river water protozoa and competition by native bacteria have also had an effect.

13. The complex interactions among the indigenous aquatic microorganisms and *R. solanacearum* ph II race 3 bv 2 in the water microcosms have been more rapid and intense at the temperature of 24°C than at 14°C. Therefore, 14°C has proved to be more favourable than 24°C for the survival of the pathogen in the presence of active microbiota.

14. Bacteriophages able to lysis *R. solanacearum* ph II race 3 bv 2 have been detected and isolated from surface run off waters. Their capacity to effectively destroy cells of the pathogen has been precisely demonstrated at both, 24°C and 14°C, in river water microcosms where selected phage populations caused significant decreases in the populations of the pathogen.

15. Phage resistant variants of *R. solanacearum* ph II race 3 bv 2 have appeared in the microcosms in response to the stress by the presence of river water bacteriophages. However, these forms of resistance of the pathogen were not PC-type, since they produced fluidal colonies on plates.

16. Regardless of their cold or warm origin, *R. solanacearum* ph II race 3 bv 2 introduced to temperate areas have similarly coped with indigenous microbiota, extended oligotrophy and environmental temperatures, having been able to survive, adapt to and keep pathogenic in almost all condition.

This research work provides with a greater understanding of the behaviour of *R. solanacearum* ph II race 3 bv 2 *in planta* and the survival abilities of the pathogen in water systems. From now on, any plant species can be classified into distinct categories with respect to the interaction with the pathogen, which is useful in several ways already mentioned in this work. In water systems, the bacterium has developed different strategies to adapt to various stress conditions, which turns out the implementation of any kind of efficient control treatment aimed to a total eradication of the pathogen to be very difficult into so complex a natural environment. However, this research points out to the possibility that, in natural settings, population densities of *R. solanacearum* ph II race 3 bv 2 could be effectively reduced, and therefore the survival of the pathogen limited, in the field and water systems by either the use of suitable candidates for crop rotation or the management of components of the water microbiota. New research in both topics can be now undertaken.

PART 5
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