Universitat de Girona

CHARACTERIZATION AND EFFICACY OF BACTERIAL STRAINS FOR BIOLOGICAL CONTROL OF SOIL-BORNE DISEASES CAUSED BY *Phytophthora cactorum* AND *Meloidogyne javanica* ON ROSACEOUS PLANTS

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ISBN: 978-84-691-2575-5
Dipòsit legal: GI-244-2008

Universitat de Girona

Doctoral Thesis

Characterization and Efficacy of Bacterial Strains for Biological Control of Soil-borne Diseases Caused by *Phytophthora cactorum* and *Meloidogyne javanica* on Rosaceous Plants

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Novembre 2007

Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària
Institut de Tecnologia Agroalimentària

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Memòria presentada per Lourdes Agustí Alcals, inscrita al programa de doctorat de Ciències Experimentals i de la Salut, itinerari Biotecnologia, per optar al grau de Doctor per la Universitat de Girona

Lourdes Agustí Alcals
2007

Anna Bonaterra Carreras i **Concepció Moragrega Garcia**, professores titulars de l'àrea de Producció Vegetal del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona

CERTIFIQUEN

Que la llicenciada en Biologia Lourdes Agustí Alcals ha dut a terme, sota la seva direcció, el treball amb el títol "Characterization and efficacy of bacterial strains for biological control of soil-borne diseases caused by *Phytophthora cactorum* and *Meloidogyne javanica* on Rosaceous plants", que presenta en aquesta memòria la qual constitueix la seva Tesi per a optar al grau de Doctor per la Universitat de Girona.

I per a què consti als efectes oportuns, signen la present a Girona, el 21 de novembre del 2007

Vist-i-plau de les directores de la Tesi

Dra. Anna Bonaterra Carreras

Dra. Concepció Moragrega Garcia

Emili Montesinos Seguí, catedràtic de l'àrea de Producció Vegetal del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona

Lourdes Agustí Alcals que ha inscrit la tesi doctoral titulada "Characterization and efficacy of bacterial strains for biological control of soil-borne diseases caused by *Phytophthora cactorum* and *Meloidogyne javanica* on Rosaceous plants" amb el número de registre 680 (22 d'octubre del 2004)

DECLAREN

Que aquesta tesi està sotmesa a la propietat intel·lectual compartida amb els investigadors del grup de Patologia Vegetal de la Universitat de Girona (Article 2. Apartat 2, RD 1326/2003 de 24-10-2003; Llei de la Propietat Intel·lectual, RD 1/1996 de 12-04-1996).

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Vist-i-plau

Dr. Emili Montesinos Seguí

Lourdes Agustí Alcals

Aquesta tesi ha estat parcialment subvencionada per una beca pre-doctoral FI del Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya, amb el número d'expedient 2003FI 00861.

Agraïments

Vull agraïr a totes les persones que han participat en l'el·laboració d'aquest treball la seva aportació i ajuda.

Vull donar les gràcies a les meves directores de tesi, la Dra. Anna Bonaterra i la Dra. Concepció Moragrega, per la seva gran ajuda, dedicació i paciència tant en la part experimental de la tesi com en l'el·laboració d'aquest manuscrit. He tingut molta sort d'haver-vos tingut de directores i he après moltes coses durant aquests anys gràcies a vosaltres.

Gràcies al Dr. Emili Montesinos per haver-me donat la possibilitat de formar part del seu grup i per haver-me permès utilitzar les instal·lacions i el material necessari durant tot el període de doctorat.

Gràcies a tots els companys i companyes que heu compartit amb mi tots aquests anys de tesi:
A la Rosalia, amb qui tantes hores de laboratori i de despatx he passat, parlant d'això, d'allò i de moltes altres coses, i amb qui he pogut xerrar de cosetes toves desinhibidament. Gràcies per aguantar-me!
A l'Olga, que tantes coses m'ha ensenyat i amb qui tantes bones estones he passat. Molta feina de laboratori hagués estat molt més difícil i pesada sense la teva ajuda. Gràcies per tot!
A l'Albert i la Gemma, que tot i que estan més hores treballant que dormint no han perdut el salero. Ànims i molta sort!
A la Mari, la Marta (les meves companyes pintores), la Nuri i l'Eduard, que també han hagut de passar pel mateix. A la Lídia, l'Isidre, l'Esther, en Jordi, en Jesús, en Josep, en Pere i tots els nois i noies que han passat per l'hivernacle o el laboratori i que m'han donat molts cops de ma: la Gàlia, l'Anna, l'Anna Viladecans, en Ferran i en David Serra. Gràcies també a la Patri, la Mònica Ortiz i la Roser que ja no estan a la uni però que també m'han ajudat més d'un cop i amb qui he passat molt bones estones.
A tothom amb qui he compartit més d'un àpat al menjador i que també m'han ajudat molts cops amb una cosa o altra: la Nuri, la Gemma, l'Elena, la Dolors, la Mònica, l'Anna Maria, en Pere, la Susanna, la Maria, l'Anna Nadal i l'Anna Coll. Gràcies per tot!

Vull agraïr al Dr. Jaume Camps la seva col·laboració en la detecció de metabolits per HPLC i NMR.

També vull agraïr el servei, l'ajuda i l'amabilitat de la Carme Carulla, en Jordi Blavia, en Dani i l'Imma, del Servei de Microscopia de la Universitat de Girona.

Finalmente, quiero dar las gracias al Dr. Jorge Pinochet, por su colaboración, sus consejos y por habernos proporcionado las poblaciones de nematodos y los portainjertos GF-677.

Als meus pares

A en Manel

Summary

Biological control of plant diseases caused by soil-borne pathogens is an alternative to chemical control with applicability in Integrated Pest Management (IPM). However, the main problem of biological control is its low consistency and reliability under field conditions due to a high variability in efficacy. In this study, 58 *Pseudomonas fluorescens* and *Pantoea agglomerans* strains were evaluated. *P. fluorescens* strains that reduced disease caused by the Oomycete *Phytophthora cactorum* (Lebert & Cohn) J. Schröter in strawberry plants and by the root-knot nematode *Meloidogyne javanica* (Treub) Chitwood in the peach-almond hybrid (*Prunus persica x P. dulcis*) GF-677 rootstock were selected, characterized and tested individually and in combination for their use in biological control of these pathogens.

A detached leaf inoculation method was developed for screening *P. fluorescens* and *P. agglomerans* strains as biological control agents of *P. cactorum* in strawberry. Optimal conditions for *P. cactorum* infection in detached leaves consisted of inoculation of zoospore suspensions from 18- to 25-day-old cultures of the pathogen on wounded young leaves from susceptible strawberry cultivars. Infection symptoms were well developed 10 days after incubation at optimal conditions. Additionally, penetration, colonization and infection of leaf tissues by *P. cactorum* was confirmed by light and transmission electron microscopy. The *ex vivo* screening of 17 bacterial strains for biological control of *P. cactorum* in strawberry permitted to range bacterial strains according to their efficacy in the reduction of the pathogen infection and to select the most effective strains for further *in planta* assays. Most bacterial strains performed similarly in disease control in both *ex vivo* and *in planta* assays, indicating that the developed detached leaf method can be used for screening biological control strains as an alternative to preliminary *in planta* assays, thus reducing time, space and plant material. *P. fluorescens* strains that reduced *P. cactorum* infection on leaves were able to colonize xylem vessels and protect leaf tissues from deterioration caused by pathogen infection.

P. fluorescens EPS599, EPS817 and EPS894 were selected from a collection of 58 *P. fluorescens* and *P. agglomerans* strains as efficient *P. cactorum* biocontrol strains since they significantly reduced infection severity on strawberry detached leaves and disease severity *in*

planta, showing different *in vitro* antagonistic activity against the pathogen. Strain EPS599 synthesized pyoluteorin (Plt) and showed chitinolytic and inorganic phosphate solubilization ability, whereas strain EPS817 synthesized hydrogen cyanide (HCN) and 2,4-diacetylphloroglucinol (DAPG), and strain EPS894 synthesized pyoluteorin and phenazine-1-carboxylic acid (PCA). All strains synthesized siderophores and produced these secondary metabolites in strawberry collar root extract (SCRE) and potato-dextrose broth (PDB). Individual and combined cell suspensions and cell-free culture extracts of these strains reduced *P. cactorum* cyst germination on SCRE. The addition of nutrients to cell-free culture extracts restored 50% of cyst germination, confirming that antibiosis was the mode of action by which *P. fluorescens* EPS599, EPS817 and EPS894 reduced cyst germination *in vitro*, and being nutrient competition only partly responsible of this reduction. Strains EPS599 and EPS894 had also a significative effect on reduction of *P. cactorum* mycelial growth on PDB. *P. fluorescens* EPS599, EPS817, EPS894 and their combinations significantly reduced Phytophthora Root Rot in strawberry. Strain mixtures seemed to improve consistency between experiments and reduce variability compared to strains applied individually.

P. fluorescens EPS384, EPS895 and CHA0 were selected from a 58-strain collection for their efficacy in reduction of gall formation and egg production on GF-677 inoculated with the root-knot nematode *M. javanica*, and for their overall metabolic and *in vitro* antagonistic activity against other plant pathogens. All three strains showed a high biocontrol efficacy and different levels of metabolic activity and *in vitro* antagonism. Strain EPS384 synthesized siderophores, salicylic acid (SA), and showed inorganic phosphate solubilization ability. Strain EPS895 synthesized siderophores and pyoluteorin, and even though genes related to PCA synthesis were detected in this strain, production of PCA was not detected in LB medium. Finally, *P. fluorescens* CHA0, which was selected as a biocontrol reference, synthesized siderophores, HCN, 1-indole-3-acetic acid (IAA), DAPG, SA, pyrrolnitrin (Prn) and pyoluteorin, and presented a capacity to hydrolyse chitin and solubilize inorganic phosphate. *P. fluorescens* EPS384, EPS895 and CHA0 reduced juvenile survival in liquid medium *in vitro*, and strains EPS384 and CHA0 also reduced *M. javanica* egg hatching under these conditions, whereas no effect was observed in solid substrate *in vitro*. *P. fluorescens* EPS384, EPS895 and their combination significantly reduced root galling on *M. javanica*-inoculated GF-677. The combination of strains did not increase biocontrol efficacy, but it seemed to reduce variability between experiments, thus increasing consistency. Additionally, since strain EPS384 synthesized SA, a split-root test was performed

to assess whether the control mechanism of this strain was induction of plant resistance. This assay confirmed that EPS384 might induce systemic resistance in the plant, thus reducing *M. javanica* infection.

Resum

El control biològic de les malalties de les plantes causades per patògens del sòl és una alternativa al control químic amb aplicabilitat en el Control Integrat de Malalties. Tanmateix, el problema principal del control biològic és la seva baixa consistència i fiabilitat en condicions de camp a causa de l'alta variabilitat en l'eficàcia. En aquest estudi s'avaluaren 58 soques de *Pseudomonas fluorescens* i *Pantoea agglomerans*. Les soques de *P. fluorescens* que reduiren la malaltia causada per l'Oomicet *Phytophthora cactorum* (Lebert & Cohn) J. Schröter en maduixera i pel nematode formador de gal·les *Meloidogyne javanica* (Treub) Chitwood en el portaempelt híbrid de presseguer i ametller (*Prunus persica x P. dulcis*) GF-677 es van seleccionar, caracteritzar i provar individualment i en combinació per tal de ser utilitzades en el control biològic d'aquests patogens.

Es desenvolupà un mètode *ex vivo* d'inoculació de fulla amb l'objectiu de seleccionar soques de *P. fluorescens* i *P. agglomerans* com a agents de control biològic de *P. cactorum* en maduixera. Les condicions òptimes per produir infecció de *P. cactorum* en fulla en condicions *ex vivo* s'obtingueren mitjançant la inoculació de suspensions de zoòspores obtingudes de cultius de 18 a 25 dies del patogen sobre ferida en fulles joves de varietats susceptibles de maduixera. Els símptomes de la infecció es desenvoluparen després de 10 dies d'incubació en condicions òptimes. Addicionalment, es confirmà la capacitat de *P. cactorum* de penetrar, colonitzar i infectar els teixits de la fulla mitjançant microscopia òptica i electrònica de transmissió. La selecció en condicions *ex vivo* de 17 soques bacterianes pel control biològic de *P. cactorum* en maduixera permeté distribuir les soques bacterianes segons la seva eficàcia en la reducció de la infecció i seleccionar les soques més eficaces per posteriorment realitzar altres assajos *in planta*. Moltes soques bacterianes mostraren un control de la malaltia similar tant en assajos *ex vivo* com *in planta*, indicant que el mètode *ex vivo* d'inoculació de fulla es pot utilitzar per seleccionar soques de biocontrol i com a alternativa als assajos preliminars en planta, reduint d'aquesta manera temps, espai i material vegetal. Les soques de *P. fluorescens* que reduien la infecció de *P. cactorum* en fulla podien colonitzar els vasos del xilema i protegir els teixits de la fulla de la deterioració causada per la infecció del patogen.

P. fluorescens EPS599, EPS817 i EPS894 es seleccionaren a partir d'una col·lecció de 58 soques de *P. fluorescens* i *P. agglomerans* com a soques eficaces en el biocontrol de *P. cactorum* ja que reduiren significativament la severitat de la infecció en fulles de maduixera inoculades amb *P. cactorum*, mostraren diferent activitat antagonista *in vitro* enfront el patogen i també reduiren significativament la severitat de la malaltia en maduixera. La soca EPS599 sintetitza pioluteorina (Plt) i mostra activitats quitinolítica i solubilitzadora de fosfats inorgànics, mentre que la soca EPS817 sintetitza àcid cianhídric (HCN) i 2,4-diacetilfloroglucinol (DAPG), i la soca EPS894 sintetitza pioluteorina i àcid fenazin-1-carboxílic (PCA). Totes les soques sintetitzen sideròfors i produeixen aquests metabolits tant en extracte de coll d'arrel de maduixera (SCRE) com en brou patata-dextrosa (PDB). Les cèl·lules i els extractes estèrils de cultius d'aquestes soques reduiren, tant individualment com en combinació, la germinació de cists de *P. cactorum* en SCRE, mentre que l'addició de nutrients als extractes estèrils de cultius només permeté recuperar un 50% de la germinació dels cists, confirmant així que l'antibiosi és el mecanisme d'acció pel qual *P. fluorescens* EPS599, EPS817 i EPS894 redueixen la germinació dels cists, i essent la competició per nutrients només parcialment responsable d'aquesta reducció. Les soques EPS599 i EPS894 també mostraren un efecte significatiu en la reducció del creixement del miceli de *P. cactorum* en PDB. *P. fluorescens* EPS599, EPS817, EPS894 i les seves combinacions reduiren la severitat de la malaltia en maduixera. La combinació de soques semblà millorar la consistència entre experiments i reduir la variabilitat en comparació amb les soques aplicades individualment.

P. fluorescens EPS384, EPS895 i CHA0 es seleccionaren a partir d'una col·lecció de 58 soques per la seva eficàcia en la reducció de la formació de gal·les i la producció d'ous en portaempelts GF-677 inoculats amb el nematode *M. javanica*, i per la seva activitat antagonista *in vitro* enfront altres patògens de plantes i la seva activitat metabòlica global. Les tres soques mostraren una alta eficàcia en el biocontrol i diferents nivells d'activitat antagonista *in vitro* i activitat metabòlica. La soca EPS384 sintetitza sideròfors, àcid salicílic (SA) i mostra activitat solubilitzadora de fosfats inorgànics. La soca EPS895 sintetitza sideròfors i pioluteorina i, tot i que s'havien detectat gens relacionats amb la síntesi de PCA, la producció de PCA no es detectà en medi LB. *P. fluorescens* CHA0, la qual es seleccionà com a agent de biocontrol de referència, sintetitza sideròfors, HCN, àcid 1-indol-3-acètic (IAA), DAPG, SA, pirrolnitrina (Prn) i pioluteorina, i presenta activitat quitinolítica i solubilitzadora de fosfats inorgànics. *P. fluorescens* EPS384, EPS895 i CHA0 reduiren la supervivència de juvenils en medi líquid *in vitro*, i les

soques EPS384 i CHA0 reduiren l'eclosió d'ous de *M. javanica* en les mateixes condicions, mentre que no s'observà cap efecte de les soques sobre el nematode en substrat sòlid *in vitro*. *P. fluorescens* EPS384, EPS895 i la seva combinació reduiren significativament l'agallament de les arrels de GF-677 inoculats amb *M. javanica*. La combinació de soques no incrementà l'eficàcia del biocontrol, però semblà reduir la variabilitat entre experiments, incrementant així la consistència del biocontrol. Addicionalment, es realitzà un assaig "split-root" amb la soca EPS384 productora de SA per tal de comprovar si el mecanisme de control d'aquesta soca podria ser la inducció de resistència en la planta. Aquest assaig confirmà que EPS384 podria induir la resistència sistèmica de la planta, podent així reduir la infecció de *M. javanica*.

Resumen

El control biológico de las enfermedades de las plantas causadas por patógenos del suelo es una alternativa al control químico aplicable en el Control Integrado de Enfermedades. Sin embargo, el principal problema del control biológico es su baja consistencia y fiabilidad en condiciones de campo debido a la elevada variabilidad en la eficacia. En este estudio se evaluaron 58 cepas de *Pseudomonas fluorescens* y *Pantoea agglomerans*. Las cepas de *P. fluorescens* que redujeron la enfermedad causada por el Oomicete *Phytophthora cactorum* (Lebert & Cohn) J. Schröter en fresón y por el nematodo agallador *Meloidogyne javanica* (Treub) Chitwood en el portainjerto híbrido de melocotonero y almendro (*Prunus persica x P. dulcis*) GF-677 se seleccionaron, caracterizaron y probaron individualmente y en combinación para ser utilizadas en el control biológico de estos patógenos.

Se desarrolló un método *ex vivo* de inoculación de hoja con el objetivo de seleccionar cepas de *P. fluorescens* y *P. agglomerans* como agentes de control biológico de *P. cactorum* en fresón. Las condiciones óptimas para producir infección de *P. cactorum* en hoja en condiciones *ex vivo* se lograron mediante la inoculación de suspensiones de zoosporas obtenidas de cultivos de 18 a 25 días del patógeno sobre herida en hojas jóvenes de variedades susceptibles de fresón. Los síntomas de la infección se desarrollaron después de 10 días de incubación en condiciones óptimas. Adicionalmente, se probó la capacidad de *P. cactorum* de penetrar, colonizar e infectar los tejidos foliares mediante microscopía óptica y electrónica de transmisión. La selección en condiciones *ex vivo* de 17 cepas bacterianas para el control biológico de *P. cactorum* en fresón permitió distribuir las cepas bacterianas según su eficacia en la reducción de la infección y seleccionar las cepas más eficaces para posteriormente realizar otros ensayos *in planta*. Muchas cepas bacterianas mostraron un control de la enfermedad similar tanto en ensayos *ex vivo* como *in planta*, indicando que el método *ex vivo* de inoculación de hoja se puede utilizar para seleccionar cepas de biocontrol y como alternativa a los ensayos en planta, reduciendo así tiempo, espacio y material vegetal. Las cepas de *P. fluorescens* que redujeron la infección de *P. cactorum* en hoja podían colonizar los vasos del xilema y proteger los tejidos foliares de la deterioración causada por la infección del patógeno.

P. fluorescens EPS599, EPS817 y EPS894 se seleccionaron a partir de una colección de 58 cepas de *P. fluorescens* y *P. agglomerans* como cepas eficaces en el biocontrol de *P. cactorum* ya que redujeron más del 40% la severidad de la infección en hojas de fresón inoculadas con *P. cactorum*, mostraron diferente actividad antagonista *in vitro* frente al patógeno y redujeron la severidad de la enfermedad en fresón. La cepa EPS599 sintetiza pioluteorina (Plt) y muestra actividades quitinolítica y solubilizadora de fosfatos inorgánicos, mientras que la cepa EPS817 sintetiza ácido cianhídrico (HCN) y 2,4-diacetilfloroglucinol (DAPG), y la cepa EPS894 sintetiza pioluteorina y ácido fenazin-1-carboxílico (PCA). Todas las cepas sintetizan sideróforos y producen estos metabolitos en extracto de cuello de raíz de fresón (SCRE) y en caldo patata-dextrosa (PDB). Las células y los extractos estériles de cultivos de estas cepas, tanto individualmente como en combinación, redujeron la germinación de *P. cactorum* en SCRE, mientras que la adición de nutrientes en los extractos estériles de cultivos solamente permitió recuperar un 50% de la germinación, confirmando así que la antibiosis era el mecanismo de acción mediante el cual *P. fluorescens* EPS599, EPS817 y EPS894 redujeron la germinación del patógeno, siendo la competición por nutrientes sólo parcialmente responsable de esta reducción. Las cepas EPS599 y EPS894 también mostraron un efecto significativo en la reducción del crecimiento del micelio de *P. cactorum* en PDB. *P. fluorescens* EPS599, EPS817, EPS894 y sus combinaciones redujeron significativamente la severidad de la enfermedad en fresón. Las combinaciones de cepas parecieron mejorar la consistencia entre experimentos, reduciendo la variabilidad, en comparación con las cepas aplicadas individualmente.

P. fluorescens EPS384, EPS895 y CHA0 se seleccionaron a partir de una colección de 58 cepas por su eficacia en la reducción de la formación de agallas y la producción de huevos en portainjerto GF-677 inoculado con el nematodo agallador *M. javanica*, así como por su comportamiento antagonista *in vitro* frente a otros patógenos de plantas y su actividad metabólica global. Las tres cepas mostraron una elevada eficacia en el biocontrol y diferentes niveles de actividad antagonista *in vitro* y actividad metabólica. La cepa EPS384 sintetiza sideróforos, ácido salicílico (SA) y muestra actividad solubilizadora de fosfatos inorgánicos. La cepa EPS895 sintetiza sideróforos y pioluteorina y, aunque se habían detectado genes relacionados con la síntesis de PCA, la producción de PCA no se detectó en medio LB. *P. fluorescens* CHA0, que se seleccionó como agente de biocontrol de referencia, sintetiza sideróforos, HCN, ácido 1-indol-3-acético (IAA), DAPG, SA, pirrolnitrina (Prn) y pioluteorina, y presenta actividad quitinolítica y solubilizadora de fosfatos inorgánicos. *P. fluorescens* EPS384,

EPS895 y CHA0 redujeron la supervivencia de juveniles en medio líquido *in vitro*, y las cepas EPS384 y CHA0 redujeron la eclosión de huevos de *M. javanica* en las mismas condiciones, mientras que no se observó efecto de las cepas sobre el nematodo en sustrato sólido *in vitro*. *P. fluorescens* EPS384, EPS895 y su combinación redujeron significativamente el agallamiento de las raíces de GF-677 inoculado con *M. javanica*. La combinación de cepas no incrementó la eficacia del biocontrol, pero pareció reducir la variabilidad entre experimentos, incrementando así la consistencia de biocontrol. Adicionalmente se realizó un ensayo "split-root" con la cepa EPS384 productora de SA para comprobar si el mecanismo de control de esta cepa podría ser la inducción de resistencia en la planta. Este ensayo confirmó que EPS384 podría inducir la resistencia sistémica de la planta, pudiendo de esta manera reducir la infección de *M. javanica*.

List of abbreviations

ANOVA	Analysis of variance
AZCL	Azurine-dyed cross-linked substrate
BCA	Biological control agent
CAS	Chrome azurol S
DAPG	2,4-diacetylphloroglucinol
GMM	Glucose minimum medium
HCN	Hydrogen cyanide
HPLC	High performance liquid chromatography
HSD	Tukey's studentized range test
IAA	1-indole-3-acetic acid
ISR	Induced systemic resistance
J2	Second-stage juvenile
LB	Luria-Bertani medium
LSD	Fisher's least significant test
MAPG	Monoacetylphloroglucinol
MH	Müeller-Hinton medium
NBRIP	National Botanical Research Institute's phosphate growth medium
NBRIY	National Botanical Research Institute's phosphate growth medium devoid of yeast extract
NMR	Nuclear magnetic ressonance
PC	Principal components (analysis). Also abbreviated as PCA in literature.
PCA	Phenazine-1-carboxylic acid
PCR	Polymerase chain reaction
PDA	Potato-dextrose agar
PDB	Potato-dextrose broth
Plt	Pyoluteorin
Prn	Pyrrolnitrin
RAPD	Random amplified polymorphic DNA
RI	Reproduction index
SA	Salicylic acid
SAR	Systemic acquired resistance
SCRE	Strawberry collar root extract
SEM	Scanning electron microscopy
Sid	Siderophores
SSM	Standard succinate medium
TEM	Transmission electron microscopy
TSA	Triptone-soy agar
V8	Eight-vegetables medium

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General Introduction

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Control methods of soil-borne plant diseases

In the last decades, chemical pesticides have been used to diminish crop and yield loses caused by plant pathogens and pests, and at the same time, to increase yield and economic gains (Lumsden *et al.*, 1995). However, the continuous and abusive application of chemical products has lead to the apparition of environmental and human health problems. Some of these negative effects are the accumulation of chemical pesticides in plants and crop products, contamination of the environment and water resources, the reduction or elimination of beneficial organisms that naturally control some pathogens, and the selection of resistant pathogens (Kuhajek *et al.*, 2003).

In the last years, the number of registered chemical pesticides has decreased as a result of the restrictions imposed because of their negative effects on human health and the environment (Sutton, 1996), and with the objective to reduce the residual levels of these chemicals on food, soils and water resources (Ragsdale and Sisler, 1994).

To reduce chemical pesticide usage, efforts must be put on research for alternative or complementary control methods. Some methods have already been studied and applied successfully in several crops. One example of a chemical control method that has been displaced by other less harmful chemical products and control practices is soil fumigation with methyl bromyde. Methyl bromyde is a biocide gas with a wide action range that is able to

penetrate into the soil, being very effective at low concentrations. However, it is difficult to degradate and it excessively accumulates in treated plants. Methyl bromide, which has a half-life in the atmosphere of 2 years, has also negative effects on the environment as it is one of the products responsible of the deteriodation of the ozone layer. For all these reasons, the use of methyl bromide in agriculture has been prohibited since 2005 in all countries that signed the Montreal Protocol (Montreal Protocol, 1989, Ozone Secretariat, United Nations Environment Programme (UNEP)). Some alternative products to methyl bromide have been used in different crops, especially in strawberry fields, such as chloropicrin, 1,3-dichloropropene, methyl isothiocyanate (metam sodium or dazomet), methyl iodide and propargyl bromide. Some of these soil fumigants are more or less effective against different soil-borne pathogens and weeds, and are usually applied combined to enhance their efficacy (Martin, 2003). Other alternatives comprise the use of natural products to stimulate plant defenses, cultural practices and biological control. These alternative control methods and a rational use of less harmful chemical products constitute the integrated control of plant pathogens, which is the best alternative to the indiscriminate application of chemical products that has been the major control practice in agriculture for a longtime.

Alternative methods in control of soil-borne diseases include stimulation of plant defenses, cultural practices and biological control. Plant defense inducers are natural products such as plant extracts (e.g. neem oil), algal extracts (e.g. laminarin), secondary metabolites produced by microorganisms (e.g. harpin) and essential oils (e.g. geraniol, menthol) that, instead of having a direct effect on pathogens, act by the stimulation and reinforcement of plant defenses, causing a minor impact on the soil ecology. A problem of these natural products is that they are usually composed by many different molecules, and the composition of a given extract might differ from another depending on the extraction method or the source plant used.

Different cultural practices, such as using healthy seeds and transplants, growing seedlings in pathogen-free peat or rockwool plugs, installing nets to avoid entrance of insects in a greenhouse, disinfestation of nutrient solutions, elimination of diseased plants, cleaning all equipment before entering a new field, crop rotation, and plant residues management, can contribute to reduce populations of soil-borne pathogens. Solarization and biofumigation or biodisinfection have proved to be effective and are being used as preventive techniques before planting. Solarization leads to a decrease of pathogen populations in favour of beneficial microorganisms and it is especially effective in warm and sunny conditions such as those of Mediterranean climate. Biofumigation or biodisinfection are used in cooler climates, and are

based on plastic tarping of the soil after the incorporation of fresh organic matter that fermentates, producing toxic metabolites as well as anaerobic conditions. Compost amendments are also used to control soil-borne pathogens, but their efficacy depends on the source used, the composting process, the microorganisms that colonize the compost and the target pathogens (Alabouvette *et al*., 2006).

Finally, biological control, especially microbiological control of plant pathogens, has a high potential to be used for soil-borne diseases and is described below.

Biological control of soil-borne pathogens

Biological control of plant diseases can be defined as the use of beneficial microorganisms to control aerial or soil-borne plant pathogens. In comparison with chemical control or biological control of insect pests, biological control of diseases caused by plant pathogens is still not fully developed, although more and more research and efforts are invested on it because of its high potential as a control method. Currently, several biological control products are commercially available in different countries, especially in the USA, Australia and New Zealand, where registration of these products is more flexible than in the European Union (Table 1). In 1991, the European Union created the directive 91/414/EEC for the sustainable usage of chemical pesticides. This directive also comprises the only accepted and registered microbial pesticides for use in the EU. Only six biological control agents (BCA) are listed in Annex 1 of this directive: *Ampelomyces quisqualis* strain AQ10, *Coniothyrium minitans*, *Bacillus subtilis* QST7B, *Paecilomyces fumosoroseus*, *Apopka* sp. strain 97, *Pseudomonas chlororaphis* strain MA342 and *Gliocladium catenulatum* strain J1446, among others pending of decisions (Alabouvette *et al*., 2006; European Union, 2007a; European Union, 2007b).

Before a microbial pesticide, especially a bacterial strain, can be registered and commercialized several steps must be fulfilled. Potentially antagonistic bacteria should be isolated in pure culture from samples of soil or plant material obtained from disease-suppressive soils or healthy plants in epidemic areas. These bacterial isolates must be identified, characterized and tested for control efficacy in bioassays in presence of the target pathogen *in vitro*, *ex vivo* or *in vivo*. Afterwards, trials must be performed under field or greenhouse conditions to assess control efficacy of the bacterial strains tested under such conditions. Screening for antagonistic bacteria against a soil-borne pathogen is a critical step, and selection criteria for biological control agents (BCA) depend on the screening method.

Table 1. Biological control agents commercially available in several countries and target soil-borne pathogens. Source: APS Biological Control Commitee, 2005; Database of Microbial Biopesticides, 2005.

Biological control agent	Trade name	Manufacturer	Target pathogens	Host plants
Agrobacterium radiobacter K1026	Nogall™	Becker Underwood Pty., Ltd. (Australia); New Bioproducts, Inc. (USA)	*Agrobacterium tumefaciens*	Fruit and nut trees Caneberries Roses and other ornamental nursery stock
Agrobacterium radiobacter 84	Galltrol™	AgBioChem, Inc. (USA)	*Agrobacterium tumefaciens*	Fruit and nut trees Ornamental nursery stock
Bacillus pumilus GB34	GB34™ YieldShield™	Gustafson, Inc. (USA)	*Fusarium* spp. *Rhizoctonia* spp. and other root pathogens	Soybean
Bacillus subtilis MBI600	Subtilex™	Becker Underwood, Ltd. (USA)	*Fusarium* spp. *Rhizoctonia* spp. *Pythium* spp.	Field, ornamental and vegetable crops
Bacillus subtilis MBI600 + rhizobia	HiStick N/T™	Becker Underwood, Ltd. (USA)	*Fusarium* spp. *Rhizoctonia* spp. *Aspergillus* spp.	Soybean Alfalfa Peanut and others
Bacillus subtilis var. *amyloliquefaciens* FZB24	Taegro™ Tae-Technical™	EarthBiosciences Inc. (USA)	*Fusarium* spp. *Rhizoctonia* spp.	Greenhouse crops: ornamentals and trees
Bacillus subtilis GB03 + other strains of *B. Subtilis, B. Lichenformis, B. megaterium*	Companion™	Growth Products, Ltd. (USA)	*Fusarium* spp. *Rhizoctonia* spp. *Pythium* spp. *Phytophthora* spp.	Greenhouse and nursery crops
Bacillus subtilis GB03 (different formulations)	Kodiak™ Kodiak HB™ Epic™ Concentrate™ Quantum 4000™ System 3™	Gustafson, Inc. (USA)	*Rhizoctonia solani* *Fusarium* spp. *Alternaria* spp. *Aspergillus* spp. and other root pathogens	Greenhouse and nursery crops
Burkholderia cepacia (var. Wisconsin)	Deny™ Blue Circle™ Intercept™	Helena Chemical Company (USA); Stine Microbial Products (USA); Soil Technologies (USA)	*Fusarium* spp. *Rhizoctonia* spp. *Pythium* spp. Nematodes	Alfalfa Barley Cotton Wheat and other vegetable crops
Gliocladium spp.	Gliomix™	Verdera Oy. (Finland)	*Rhizoctonia solani* *Pythium* spp.	Greenhouse and nursery crops
Gliocladium catenulatum	Primastop™	Kemira Agro Oy. (Finland); AgBio Development, Inc. (USA)	Soil-borne pathogens	Ornamental, vegetable and tree crops

Table 1. Continuation.

Biological control agent	Trade name	Manufacturer	Target pathogens	Host plants
Gliocladium virens GL-21	SoilGuard™	Certis USA LLC. (USA); Thermo Trilogy (USA)	*Rhizoctonia solani* *Pythium* spp. and other root pathogens	Ornamental and food greenhouse and nursery crops
Paecilomyces lilacinus 251	MeloCon WG™	Prophyta Biologischer Pflanzenschutz (Germany); WF Storeman Company LLC. (USA)	Plant parasitic nematodes	Many crops
Pseudomonas chlororaphis 63-28	AtEze™	Turf Sciences Laboratories, Inc. (USA); EcoSoil Systems, Inc. (USA)	*Rhizoctonia solani* *Fusarium oxysporum* *Pythium* spp. and other wilt diseases, stem and root rots	Ornamentals and vegetables grown in greenhouses
Pseudomonas spp. + *Azospirillum* spp.	BioJet™	EcoSoil Systems, Inc. (USA)	Soil pathogens that cause brown patch and dollar spot	Not specified
Pythium oligandrum	Polygandron™	Plant Production Institute (Slovak Republic)	*Pythium ultimum*	Sugar beet
Serratia entomophila	Invade™	Ag Research (New Zealand)	*Meloidogyne incognita* and insects	Not specified
Streptomyces griseoviridis K61	Mycostop™	Kemira Agro Oy. (Finland); Verdera Oy. (Finland); AgBio Development, Inc. (USA)	*Fusarium* spp. *Rhizoctonia* spp. *Alternaria* spp. *Pythium* spp. *Phytophthora* spp. *Phomopsis* spp. *Botrytis* spp.	Field, ornamental and vegetable crops
Streptomyces lydicus	Actinovate™	Natural Industries, Inc. (USA)	*Fusarium* spp. *Rhizoctonia* spp. *Alternaria* spp. *Pythium* spp. *Phytophthora* spp. *Phomopsis* spp. *Botrytis* spp.	Greenhouse and nursery crops Turf
Trichoderma harzianum Rifai KRL-AG2 (T-22)	T-22G™ T-22HB™ Root Shield™ Plant Shield™ BioTrek 22G™ Supresivit™	Wilbur-Ellis, Borregaard (Norway); BioWorks, Inc. (USA)	*Rhizoctonia solani* *Fusarium* spp. *Pythium* spp. *Sclerotium* spp. *Verticillium* spp. and other root pathogens	Trees Shrubs Ornamentals Cabbage Tomato Cucumber and other crops

Combination of different screening methods may enable the selection of antagonistic bacteria that operate by multiple mechanisms of action. However, this process of selection and characterization of effective BCAs is time-consumig and rather random.

The development of a model system formed by the target pathogen and the host plant is a useful tool to study not only pathogen-host interactions but also the control efficacy of a BCA and its mechanisms of action, dose-response relationships, among others. A microorganism that has been selected for its efficacy and consistency against a pathogen must overcome some previous steps before its registration. Several toxicological tests can be performed to assess biosafety of the microorganism, such as the hypersensibility reaction test on tobacco plants and the determination of the median lethal dose (LD_{50}) on mammals. For the commercial development of a microbial pesticide, the BCA must be mass produced at industrial scale by fermentation processes, preserved and stored by different stabilizing treatments (refrigeration, freezing, lyophilization), and formulated, using biocompatible additives that increase survival and improve application. It is also necessary the development of a traceability method to specifically detect and quantify the BCA once it has been applied. These traceability methods are based on specific genotypic markers that can be detected and quantified by quantitative PCR and other molecular techniques (Montesinos, 2003).

The major problem of biological control is its lack of consistency due to variable efficacy of the BCA depending on the soil environment where the BCA is applied, the moment and the method of application, the host plant, or the pathogen species. There are different approaches to reduce this inconsistency, which are the use of genetically modified BCAs with higher efficacy, the application of mixtures of different BCAs with different mechanisms of action and different nutritional and physiological requirements, and the alteration of the environment with soil amendments that enhance survival of BCAs (Raupach and Kloepper, 1998; Spadaro and Gullino, 2005).

Mechanisms of action of biological control agents

The mechanisms involved in biological control include, among others, colonization of infection sites and competitive exclusion of the pathogen, competition for nutrients, induction of plant resistance, and secretion of inhibitory compounds and lytic enzymes. Biological control can also indirectly promote plant growth by protecting plants from pathogens (Kloepper, 1993).

Plant growth promotion and biological control are two phenomenons that are interrelated, as some strains of plant growth-promoting rhizobacteria (PGPR) can act as biological control agents, and some BCAs can suppress plant pathogens and subsequently promote plant growth (Kloepper, 1993). PGPR and other soil bacteria can promote plant growth directly by different means, such as associative N_2 fixation, promotion of mycorrhization, regulation of ethylene production in roots and phytohormones synthesis (e.g. IAA, gibberelins), decrease of heavy metal toxicity, or by increasing nutrient disponibility to the plant (e.g. inorganic phosphate solubilization). Some PGPR strains can also indirectly promote plant growth by controlling soil-borne pathogens and other deleterious soil microorganisms (Figure 1). The ability to promote plant growth and/or to control plant pathogens, as well as the mechanisms involved in biological control, are all strain-specific and depend on the host plant, the pathogen and several environmental factors.

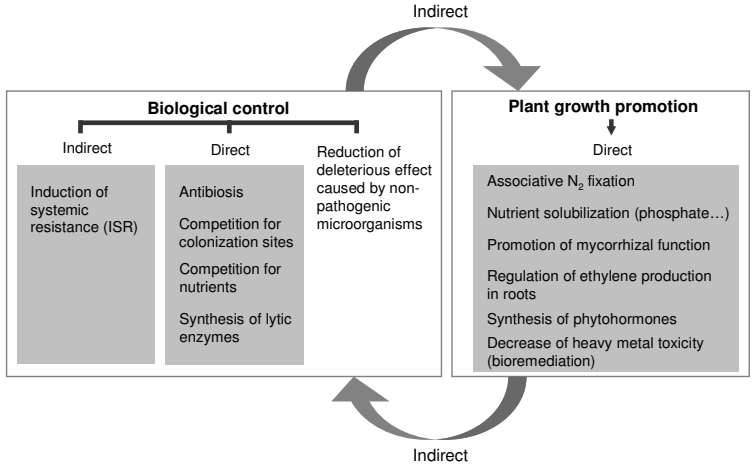

Figure 1. Relationship between biological control and plant growth promotion.

Root colonization and competition for infection sites

Rhizobacteria are bacteria with the ability to colonize the rhizosphere. Some rhizobacteria are able to colonize the root surface (rhizoplane), and others even the interior of the root and plant tissues. The latter are known as endocolonizers or endophytes. This colonization ability is called rhizosphere competence and it varies among bacterial strains (Weller, 1988; Kloepper, 1993).

Root colonization is an active process that comprises attachment and distribution of the introduced bacterial strain along the root surface, and its multiplication and survival in the

presence of indigenous rhizosphere microorganisms for several weeks. The introduced strain must be able to compete with other microorganisms, including plant pathogens, for the limited colonizable space and available nutrients. In general, common traits of root colonizers are, although not indispensable, osmotolerance, motility (flagella), fast growth rate, ability to synthesize amino acids and vitamins, ability to use root exudates (amino acids, organic acids, sugars) as nutrients or chemoattractants, and presence of different structures for adhesion to the root surface, such as pili (type IV), fimbriae, major outer membrane proteins (OprF, aglutinin), or the O-antigen chain of lipopolysaccharides (Weller, 1988; Kloepper, 1993; De Weger *et al.*, 1995; Lugtenberg and Dekkers, 1999; Compant *et al.*, 2005). Although root colonizers might be able to utilize nutrients from root exudates, concentrations of amino acids of root exudates are too low to support growth of rhizosphere microorganisms, which must be able to synthesize their own amino acids (De Weger *et al.*, 1995; Simons *et al.*, 1997). However, a correlation between the colonizing ability of a strain and its ability to use the major exudate sugars as the only carbon source has been observed. Nevertheless, utilization of root exudates is not an essential trait for root colonization and many other factors are important (Lugtenberg *et al.*, 1999).

The process of root colonization can be divided into two different phases. In phase 1 bacterial cells are attached to the root surface. As the root elongates, some of these bacteria are carried along the root tip and multiply, whereas other cells remain on older parts of the root. In phase 2, rhizosphere-competent bacteria multiply and survive on the root, competing with other rhizosphere microorganisms, including pathogens, for available nutrients. Most of the root surface remains uncolonized, and bacteria tend to form microcolonies on specific zones of the root where nutrients are more abundant (Weller, 1988; Kloepper, 1993; De Weger *et al.*, 1995; Lugtenberg and Dekkers, 1999; Gamalero *et al.*, 2004). The formation of these microcolonies may enable other mechanisms that are cell density-dependent like the production of homoserine lactones that may induce the synthesis of antibiotics and exoenzymes and, therefore, the ability of these bacteria to suppress diseases caused by plant root pathogens (Chin-A-Woeng *et al.*, 1997). Moreover, these microcolonies may develop a more structured microbial community on the rhizosphere and the root surface that can be described as a biofilm community (Davey and O'Toole, 2000). The crucial colonization level that must be reached by a BCA has been estimated at 10^5-10^6 cfu g^{-1} of root in the case of *Pseudomonas* spp., representing 0.1-1.0% of the culturable aerobic rhizobacterial population under natural conditions. Artificially introduced bacteria can initially colonize roots at 10^7-10^8 cfu g^{-1}, but these levels

always decline in a few weeks (Haas and Défago, 2005). When a bacterial strain is introduced, the total population of rhizosphere microorganisms might remain unchanged, and only the composition of the population is altered (Weller, 1988; Moënne-Loccoz *et al*., 2001).

Competition for nutrients

Competition for limited nutrients on the rhizosphere is an important mechanism of biological control agents that depends on external nutrition, but it is only relevant when the concentration of limited nutrients is low (Guetsky *et al*., 2002). Competition for nutrients in the rhizosphere and on root surfaces is more important in certain areas that are heavily colonized by microorganisms, such as cell junctions, emerging points of lateral roots and the elongation zone of the root, where concentration of root exudates and subsequent microbial growth is high (Weller, 1988). It is a general phenomenon that regulates population dynamics of microorganisms that share the same ecological niche and have the same physiological requirements, and it is based on the availability of nutrients such as carbon, nitrogen, iron, oxygen, phosphorous, and micronutrients, which in turn depends on soil temperature, pH and composition of soil and root exudates (Burr and Caesar, 1984; Handelsman and Parke, 1989; Alabouvette *et al*., 2006). Some examples of biological control by nutrient competition have been reported in literature, such as biological control of *Pythium ultimum* by *Pseudomonas fluorescen*s strain 54/96 isolated from rhizosphere (Ellis *et al*., 1999).

Even though competition for nutrients is difficult to determine and demonstrate, competition for iron has been profoundly studied and reported as one important biocontrol mechanism by rhizosphere microorganisms. Iron is essential for growth, as it is necessary for oxidative-reductive enzymatic reactions. However, it is commonly found in its insoluble form (Fe^{3+}) in neutral or alkaline soils and not available for microbial growth. Under these iron-limiting conditions, specific bacterial strains that produce iron chelating compounds like siderophores are more competitive in the rhizosphere. Siderophores are low molecular weight chelators with high affinity for Fe^{3+}. The iron-siderophore complex that is formed is recognized and taken up by specific membrane-receptor proteins located on the membrane of these bacterial strains, making Fe^{3+} unavailable to other rhizosphere microorganisms, including plant pathogens, which produce either less siderophores or different siderophores with lower binding properties. A group of rhizosphere bacteria in which siderophore-mediated biocontrol has been reported in several strains are the fluorescent pseudomonads, that produce different types of

siderophores, such as yellow-green fluorescent pyoverdins and pseudobactins (Burr and Caesar, 1984; Weller, 1988; O'Sullivan and O'Gara, 1992; Kloepper, 1993; Whipps, 2001).

Competition for nutrients is a mechanism that, although it is common, is difficult to demonstrate rigorously and therefore to exploit for biological control purposes, so it has a limited preventive effect (Jetiyanon and Kloepper, 2002; Alabouvette *et al.*, 2006). Also, it can be nullified by increasing the concentrations of the relevant nutrients in the soil (Guetsky *et al.*, 2002).

Fast-growing rhizobacteria might compete with fungal pathogens for carbon and energy sources, which would provide a basis for biological control. Although there is evidence that rhizobacteria populations can cause fungistasis in soil, and that effective biological control agents, by definition, must be able to compete for nutrients in the rhizosphere, the probability that these biocontrol agents (approximately 1% of the culturable rhizobacteria) could prevent plant disease by competition for nutrients, such as carbon sources, on the root is very low. Antibiosis, induced systemic resistance and other specific pathogen-antagonist interactions seem to be the main modes of action of biological control (Haas and Défago, 2005).

Induction of plant resistance

When a host plant is attacked by a pathogen some biochemical and physiological changes occurs that trigger a series of local defense responses that lead to a hypersensitive response (HR), subsequent cell death and formation of a necrotic lesion near the infection site. Additionally, other general resistance mechanisms induced after an HR or during pathogen infection prevent further spreading of the existing infection and combat secondary infections from a broad-spectrum of pathogens. This mechanism is also known as systemic acquired resistance (SAR), and its induction requires the signal molecule salicylic acid (SA) that accumulates in infected tissues and activates the SAR response by means of a complex molecular pathway. The SAR-induced state in plants results in expression and accumulation of pathogenesis-related (PR) proteins, production of phytoalexins, and cell wall and structural alterations. Exogenous application of SA or its functional analogs INA (2,6-dichloroisonicotinic acid) and BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester) also induces SAR.

Another plant-mediated disease suppression mechanism is induced systemic resistance (ISR). ISR is a complex process of active plant resistance and, contrary to SAR, it might be activated by exogenous application of chemical compounds different from SA that also act as signal molecules, such as jasmonic acid (JA) or ethylene, or by some non-pathogenic

rhizobacteria, resulting in an unknown molecular response different to production of PR proteins but phenotypically similar to SAR that leads to non-specific systemic resistance in the challenged plant. ISR-induced plants present some metabolic alterations that depend on the inducer rhizobacterial strain, as well as cell wall alterations similar to those of SAR-induced plants. (Kloepper *et al.*, 1992; Hoffland *et al.*, 1995; Pieterse *et al.*, 1996; Ryals *et al.*, 1996; Baker *et al.*, 1997; Van Loon, 1997; Dong, 1998; Van Loon *et al.*, 1998; Han *et al.*, 2000; Pieterse *et al.*, 2000; Whipps, 2001).

Some examples of biocontrol agents, especially fluorescent pseudomonads, that control pathogens via ISR have been reported (Han *et al.*, 2000; Jetiyanon and Kloepper, 2002; Zhang *et al.*, 2002; Jeun *et al.*, 2004; Khan *et al.*, 2004). There are also reported examples of *Pseudomonas* spp. strains that induce SAR and synthesis of PR proteins (Maurhofer *et al.*, 1994; Handelsman and Stabb, 1996; Park and Kloepper, 2000). Nevertheless, the control efficacy achieved by induction of systemic resistance is highly variable (De Meyer *et al.*, 1998). To determine that induction of plant resistance is the mechanism by which a bacterial strain suppresses root diseases, this bacterial strain and the pathogen must be inoculated in the root system separately, and this can be achieved in split-root experiments (Liu *et al.*, 1995; Siddiqui and Shaukat, 2002a) or by a separate inoculation system (Leeman *et al.*, 1995). A minimum concentration of the biocontrol agent of 10^5 cfu g^{-1} root appears to be required for induction of systemic resistance in some crops (Raaijmakers *et al.*, 1995).

Production of extracellular lytic enzymes and hyperparasitism

Biological control agents that synthesize and excrete lytic enzymes may control root diseases by parasiting target fungal pathogens. Hyperparasitism is a multi-phase mechanism which consists of the attraction of the BCA to the fungal pathogen by chemotaxis, the recognition and attachment of the BCA to specific receptors on the pathogen surface and its development and propagation. Afterwards, the BCA synthesizes extracellular cell wall-degrading enzymes that lyse the hyphal cell wall, allowing the entrance of the BCA into the pathogen, or the complete lysis and destruction of the pathogen hyphae. This process leads to the expulsion of the hyphal cytoplasmic contents to the rhizosphere, and the exploitation of the released nutrients by the BCA (Elad *et al.*, 1982; Sivan and Chet, 1992; Lorito *et al.*, 1993; Whipps, 2001). These lytic enzymes are mostly inducible enzymes that hydrolyze fungal cell wall components (e.g. chitinases, glucanases, laminarinases, cellulases, proteinases) (Compant *et al.*, 2005). Fungi are the principal producers of lytic enzymes in the soil, although several bacterial strains have also

been reported as producers, such as actinomycetes strains (Valois *et al.*, 1996), *Serratia* spp. (Ordentlich *et al.*, 1988; Berg *et al.*, 2001), *Pantoea* (*Enterobacter*) *agglomerans* (Chernin *et al.*, 1995), and *Pseudomonas* spp. (Gaffney *et al.*, 1994; Nielsen *et al.*, 1998; Berg *et al.*, 2001). Lytic enzyme synthesis and secretion by an antagonist may favour other antagonistic agents by synergism, as the latter would profit from the nutrients of the lysed hyphae. Some examples of biological control of fungal pathogens by bacterial antagonists have been reported, such as biocontrol of *Phytophthora cinnamomi* with a cellulase-producing isolate of *Micromonospora carbonacea* (El-Tarabily *et al.*, 1996), biocontrol of *Phytophthora fragariae* with actinomycetes that produced β-1,3-, β-1,4-, and β-1,6-glucanases (Valois *et al.*, 1996), biocontrol of *Rhizoctonia solani* with a chitinase-producer *Pantoea agglomerans* strain (Chernin *et al.*, 1995), and biocontrol of *Fusarium oxysporum* with combinations of bacterial strains of *Paenibacillus* spp. and *Streptomyces* spp. that produced chitinase and β-1,3-glucanase (Singh *et al.*, 1999).

Production of antibiotics and other secondary metabolites

Antibiotics are a chemically heterogeneous group of organic, low-molecular weight compounds produced by several microorganisms, and, at low concentrations, they are deleterious to the growth or metabolic activities of other microorganisms. Many biocontrol bacterial strains produce one or more antibiotics and secondary metabolites with antibiotic activity in the rhizosphere that are responsible of suppression of plant diseases caused by different pathogens (bacteria, fungi and nematodes). Several rhizobacteria and bacterial epiphytes of plants, such as the species *Pseudomonas fluorescens* and *Pantoea agglomerans*, produce multiple antibiotics against plant-pathogenic fungi and bacteria, and have been used as biocontrol agents of some diseases in the phyllosphere and the rhizosphere (Montesinos *et al.*, 1996). Among the antibiotic-producing rhizobacteria, *Pseudomonas* spp. has been the most studied since effective biocontrol strains of this genera, with relatively few exceptions, produce diffusible and/or volatile antibiotic compounds that have proved to inhibit pathogens *in vitro* and *in situ* (Raaijmakers *et al.*, 2002; Haas and Keel, 2003), and it has been reported effective in the control of various fungal plant pathogens (Silo-Suh *et al.*, 1994; Yang *et al.*, 1994; Rodríguez and Pfender, 1997; Nielsen *et al.*, 1998; Lee *et al.*, 2003).

Some of the most important secondary metabolites produced by bacterial biocontrol agents are, among others, 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA) and other phenazines, pyoluteorin, pyrrolnitrin, hydrogen cyanide (HCN), oomycin A, viscosinamide, kanosamine and zwittermycin A. These compounds with antibiotic activity may

be involved in the suppression of several plant diseases caused by different soil-borne pathogens (O'Sullivan and O'Gara, 1992; Handelsman and Stabb, 1996; Whipps, 2001; Raaijmakers *et al.*, 2002). The most studied compounds produced by *Pseudomonas* spp. are DAPG, phenazines (e.g. PCA), pyrrolnitrin, pyoluteorin and HCN (Figure 2).

Antibiotic biosynthesis by biocontrol bacteria, and in particular *Pseudomonas* spp., in the rhizosphere is modulated by exogenous (abiotic and biotic) and endogenous factors. The abiotic factors are temperature, soil moisture and pH, which also affects the activity of the antibiotic itself, whereas the main biotic factors are root exudates. Endogenous regulation comprises the global GacA/GacS regulatory system, cell density dependent regulation via *N*-acyl homoserine lactones (quorum sensing) and regulation via sigma factors, among others (Raaijmakers *et al.*, 2002; Haas and Keel, 2003).

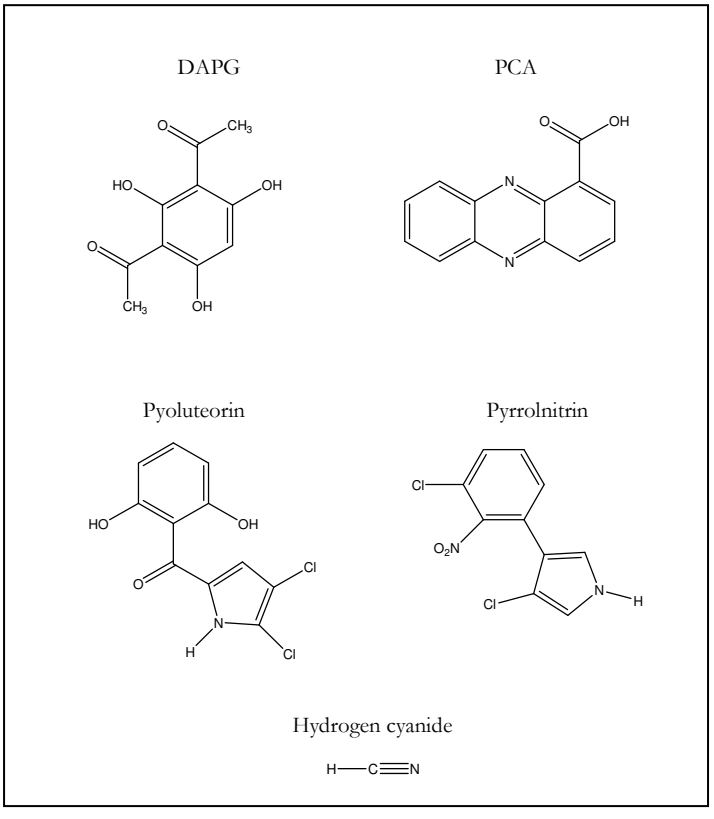

Figure 2. Secondary metabolites with antibiotic properties produced by *Pseudomonas* spp.

Improvement of the efficacy of biological control agents

The understanding of the mechanisms of action involved in biological control may enable the enhancement of control efficacy and reduce its inconsistency and variability (Guetsky *et al.*, 2002). Some previous studies suggest that the use of more than one biological control agent that operate by different mechanisms to control one or more pathogens may be a way to reduce this variability (Raupach and Kloepper, 1998; Whipps, 2001; Jetiyanon and Kloepper, 2002). Most of the soil suppressiveness of plant pathogens that can be found in crop fields may be due to naturally existing mixtures of microbial antagonists rather than high populations of a single antagonist. Application of a mixture of BCAs would be more similar to the natural situation and might permit a broader spectrum of biocontrol activity as well as an improvement of efficacy and reliability of control, and this might be due to the synergistic effect of different mechanisms of action of the introduced biocontrol strains. However, mixtures of different strains that are efficient antagonists under certain conditions might have no synergistic effect under other conditions or on different crops. For this reason, broad-spectrum BCAs may be more interesting than BCAs with a more narrow spectrum of disease protection (Ramamoorthy *et al.*, 2001). The mechanisms used by beneficial microorganisms are often difficult to determine because they can be affected by multiple factors, especially in field conditions. Some possible reasons for inconsistent performance of a given BCA are the loss of ecological competence by spontaneous mutations due to continuous growth *in vitro*, the absence of a target pathogen, interference by other non-target pathogens, or variable root colonization ability of the BCA (Weller, 1988). The use of mixtures of BCAs with different mechanisms of action reduces the possibility of resistance development in the pathogen and also allows a wider range of target pathogens in different environmental conditions (Jetiyanon and Kloepper, 2002). It is difficult to determine a single mechanism of action by which a BCA can control a pathogen, since usually more than one mechanism are interrelated. The type of BCA and the environmental factors can modify the relative importance and efficacy of each mechanism (Nielsen *et al.*, 1998). Also, the enhancement of biological control agents by means of genetic manipulation, as well as their mass production, formulation and application methods can be a way to improve biological control efficacy (Spadaro and Gullino, 2005).

Optimization of the dose and application frequency of the BCA could be another way to improve biocontrol efficacy and reduce variability. Typical application methods consist of an inundative and single application of a high BCA concentration directly in roots; however,

repetitive applications of the BCA by irrigation, resulting in its presence in the soil environment for a longer period of time, could therefore have a more durable biological control effect (Steddom *et al.*, 2002). Nevertheless, BCAs will not replace the use of chemical pesticides, although they could be an important component of integrated control strategies in agriculture (Kerry, 1997).

In conclusion, the repeated application of combinations of compatible biological control agents is a practical method to increase biocontrol efficacy and improve consistency by reducing variability, thus increasing their reliability, and which may result in a better performance when applied with other control methods in integrated pest management.

General objectives

In this study, putative biocontrol *Pseudomonas fluorescens* and *Pantoea agglomerans* strains were selected and characterized for their biocontrol efficacy against two different soil-borne pathogens in two different pathosystems: the Oomycete *Phytophthora cactorum* that infects strawberry plants causing crown and collar root rot, and the root-knot nematode *Meloidogyne javanica* that infects the GF-677 peach-almond hybrid rootstock. In each pathosystem, combinations of selected bacterial strains were tested in order to obtain compatible strain combinations that increased efficacy and/or reduced variability of biocontrol.

Chapter I

Characterization and efficacy of bacterial strains for biological control of *Phytophthora cactorum* in strawberry

Introduction

An overview of Oomycetes and *Phytophthora* spp.

The Oomycetes are a group of fungi-like mycelial organisms that belong to the Kingdom Stramenopila and represent a unique evolutionary line distant from true fungi, which belong to Kingdom Eumycota. The Oomycetes are phylogenetically more related to the golden-brown and the heterokont algae (chrysophytes and diatoms), although they are heterotrophic organisms that morfologically and physiologically resemble fungi (Sogin and Silberman, 1998; Cooke *et al.*, 2000; Kamoun, 2000; Ulloa and Hanlin, 2000; Judelson and Roberts, 2002; Tyler, 2002). Taxonomically, the Class Oomycetes is divided into three subclasses: Saprolegniomycetidae, Rhipidimiomycetidae, and Peronoporomycetidae. The latter has two Orders, Peronosporales and Pythiales. Within Pythiales is the Family Pythiaceae, where the genera *Pythium* and *Phytophthora* belong (Alexopoulos and Mims, 1985; Erwin and Ribeiro, 1996). Some of the most important plant pathogens are species of these genera.

Oomycetes, and especially *Phytophthora* species, have in common several features that differentiate them from true fungi, such as asexual reproduction and dispersion via biflagellate heterokont zoospores (one flagellum is tubular and the other has mastigonemes), sexual reproduction via formation of thick walled resistant oospores, diploid vegetative thallus, mitochondria with tubular cristae, and cell walls composed by β-1,3-glucan, β-1,6-glucan and β-1,4-glucan (cellulose). Unlike fungi, cell walls of Oomycetes generally lack chitin or contain little

amounts, depending on the species. *Phytophthora* spp. are also unable to synthesize sterols and thiamine, although they require an exogenous source of β-hydroxy sterols for sporulation and thiamine for growth. They are also resistant to toxic plant saponins, contrary to true fungi. *Phytophthora* spp. are also resistant to polyene antibiotics such as primacin (Erwin and Ribeiro, 1996; Kamoun, 2000; Kamoun, 2003; Van West *et al*., 2003).

The genus *Phytophthora* has approximately 60 species, all plant pathogens, that differ in their reproductive and infective characteristics as well as in disease development. *Phytophthora* spp., which means 'plant destroyer' in Greek, cause plant diseases in ornamental and agricultural crops, shrubs and trees on global scale, and several species have caused major disease epidemics. A very well-known example is the species *Phytophthora infestans*, responsible of the Irish potato famine (1845-1846) that caused starvation and death of over 1 million people in Ireland, and it is estimated that this species and other *Phytophthora* spp (e.g. *P. sojae, P. cactorum, P. cinnamomi*...) cost thousands of millions of dollars/euros of control measures and lost yield to commercial crops every year. Other major *Phytophthora* root pathogens are *P. palmivora* and *P. megakarya*, both tropical species that cause diseases in cocoa and are a major threat to the economies of cocoa-producing countries, and *P. ramorum*, which causes sudden death of oak trees and is currently spreading in North America and Europe. More examples of host plants and diseases caused by some of the principal *Phytophthora* species are listed in Table 1.1. Many epidemics caused by *Phytophthora* spp. are only barely controlled by the aggressive use of chemical pesticides and oomycetecides (Erwin and Ribeiro, 1996; Kamoun, 2000; Nicholls, 2004). Control methods are commented below.

The plant pathogen *Phytophthora cactorum*

Phytophthora cactorum was first reported from rotting cacti (*Melocactus nigrotomentosus* L. and *Cereus giganteus* Engelm) by Lebert and Cohn (1870). *P. cactorum* occurs worldwide but is most commonly found in temperate regions. *P. cactorum* is an heterotrophic and saprophytic soil-borne pathogen that infects more than 200 plant species and causes crown, collar and root rot, fruit rot, cankers, leaf blights, wilts and seedling blights. Although isolates of *P. cactorum* are not generally host specific, some marked differences in degree of pathogenicity to different hosts have been observed.

Table 1.1. *Phytophthora* species, some of their economically important hosts and diseases caused by the pathogen. Source: Erwin and Ribeiro, 1996; De Andrés *et al.*, 1998; Nicholls, 2004).

Species	Host plants	Symptoms and diseases
P. cactorum	Woody plants (*Quercus* sp., *Acer* sp., *Abies* sp., coniferous...) Fruit trees (*Prunus* sp., *Pyrus* sp., *Malus* sp., *Olea* sp., *Juglans* sp...) Ornamental and agricultural crops (strawberry, potato, tomato...)	Stem and bark cankers Collar, crown, stem, root and fruit rots Seedling and leaf blights
P. cambivora	Several woody plants and fruit trees (Chestnut...)	Ink disease Root rot, wilt
P. capsici	Several agricultural crops (cucumber, pea, pepper...)	Leaf blight, wilt stem, root and fruit rots
P. cinnamomi var. *cinnamomi*	Over 1000 plant species: woody plants, ornamentals, several agricultural crops	Root rot, cankers Forest dieback
P. citricola	Woody plants and fruit trees (*Citrus* sp...) Several ornamental and agricultural crops	Trunk canker, blight Root and fruit rots
P. citrophthora	Woody plants and fruit trees (*Citrus* sp...) Several ornamental and agricultural crops	Gummosis disease Cankers Fibrous root rot, crown rot, brown rot of fruits
P. cryptogea	Woody plants and fruit trees Ornamental crops Several agricultural crops (cucurbitaceae, tomato...)	Root rot Wilt
P. fragariae var. *fragariae*	Several agricultural crops (strawberry, raspberry...)	Red core or red stele Root rot
P. infestans	Some woody plants Agricultural crops (*Solanum* sp., tomato...)	Late blight of potato Leaf blight Mildew
P. megakarya	Cocoa	Black pod of cocoa
P. megasperma	Woody plants and fruit trees Some agricultural crops	Root rot Crown rot
P. nicotianae (formely *P. parasitica*)	Some woody plants and fruit trees Agricultural and ornamental crops	Brown rot of citrus Fruit, root and crown rots Gummosis Leaf blight
P. palmivora var. *palmivora*	Some woody plants cocoa	Black pod rot, fruit and root rots Seedling blight
P. ramorum	Woody plants (*Quercus* sp...) Several ornamental crops	Sudden oak death Collar and root rot
P. sojae	Soybean *Lupinus* sp.	Stem and root rots Seedling wilt
P. syringae	Several Rosaceous plants Some woody plants (*Citrus* sp...)	Fruit and root rot Blight

The vegetative hyphae of *P. cactorum* mycelia are aseptate (coenocytic) and, like other *Phytophthora* species, can produce different propagules such as sporangia, zoospores, chlamydospores and oospores, by means of which it can disperse and germinate (Figure 1.1). Sporangia (or zoosporangia) are asexual structures that contain zoospores, but they are also able to germinate in certain conditions. *P. cactorum* sporangia are caducous, papillate and with an ellipsoidal or ovoid shape and a short pedicel. Conditions of high moisture and low temperature stimulate the differentiation of the multinucleate cytoplasmic content of sporangia which, by cytoplasmic cleavage, divide into uninucleate biflagellate zoospores. These motile zoospores can be dispersed through saturated soils and running water until they find a suitable host to infect. Chlamydospores are another type of asexual propagules that can survive in soil and plant tissues for long periods of time but are only produced by some *P. cactorum* isolates and some *Phytophthora* species.

Oospores are the result of sexual reproduction, and are resistant structures that, like chlamydospores, can survive and persist for long periods of time in the soil and in infected plant tissues until environmental conditions are favourable for germination. Sexual reproduction occurs when a female oogonium is fertilized by a male antheridium, forming a thick walled oospore. In *P. cactorum*, antheridia are paragynous, that is to say, attached to the side of the oogonium, contrary to amphigynous antheridia that surround the oogonial stalk. Reproduction is homothallic, which means that it can take place without the interaction of two different thalli or mating types, contrary to other heterothallic *Phytophthora* species (e.g. *P. infestans*).

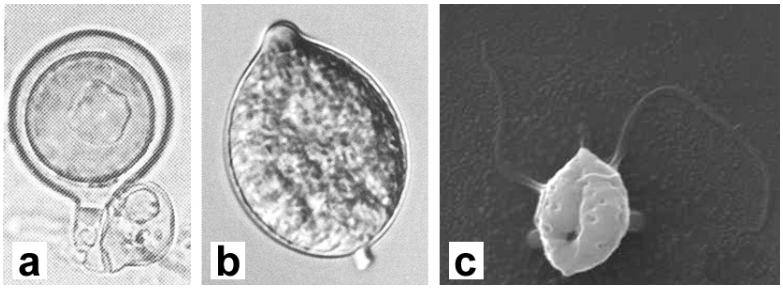

Figure 1.1. Sexual and asexual structures of *P. cactorum*. (a) Oogonium and paragynous antheridium. (b) Papillate sporangium. (c) Electronic micrograph of a zoospore showing the ventral groove and the two flagella. Source: (a, b) Erwin and Ribeiro, 1996; (c) Anonimous.

The knowledge of the life cycle of the pathogen and the processes involved in the interaction between the pathogen and the host plant is important to assess more effective disease control methods and to prevent the spread of the pathogen (Erwin and Ribeiro, 1996; Kamoun, 2000; Van West *et al.,* 2003).

Life cycle of Phytophthora cactorum

The life cycle of *P. cactorum* consists of two reproductive cycles that are usually stimulated by different environmental conditions (Figure 1.2).

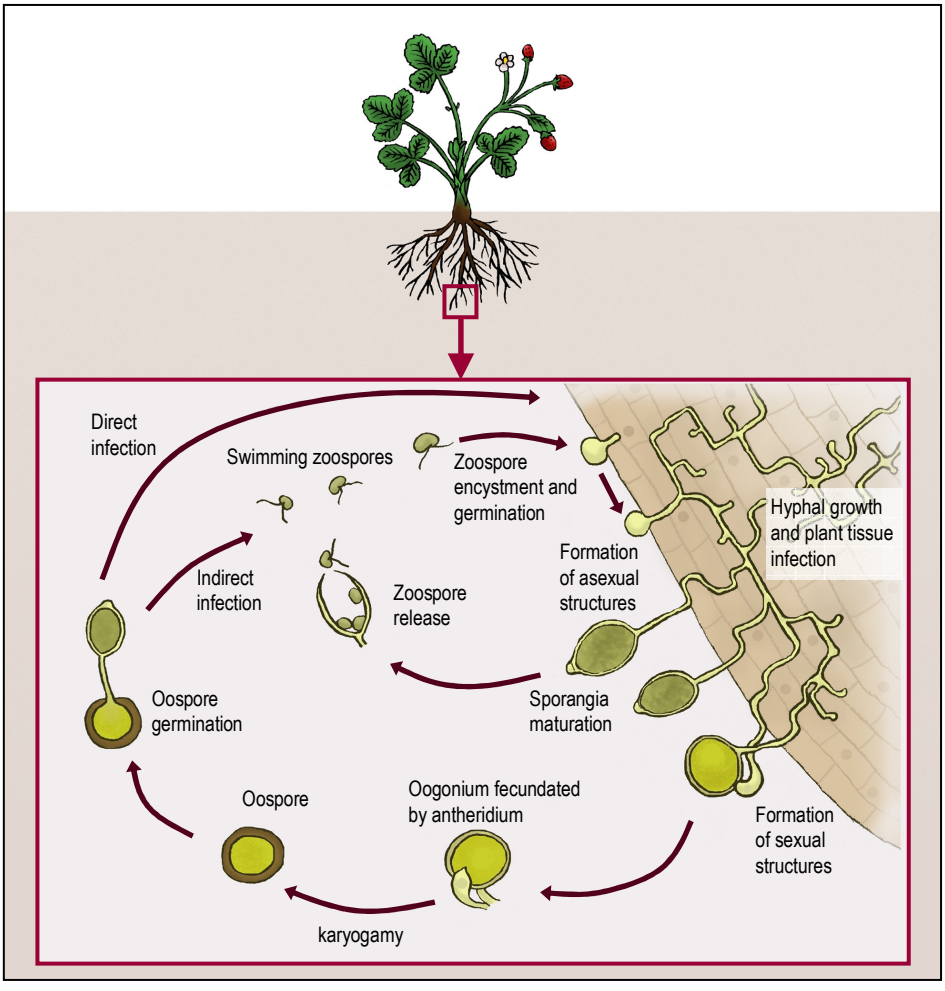

Figure 1.2. Life and disease cycle of *Phytophthora cactorum* in strawberry.

The asexual cycle is characterized by the production of sporangia in presence of water and low temperatures. The sporangia can germinate directly by production of a germ tube, or indirectly, by production of zoospores. In indirect germination, the cytoplasmic content of a mature sporangium differentiates under a cold shock stimulus, leading to cytoplasmic cleavage and hence to formation of uninucleate zoospores (approximately 10 or more). After dissolution of the papilla, zoospores are expelled from the sporangium by turgor pressure, caused by high concentrations of proline that accumulated in the cytoplasm of the cleaving sporangium. At this moment, zoospores are retained within a membranous vesicle that evaginates from the sporangium pore and eventually bursts, releasing them to the aqueous medium. Zoospores swim in search of host tissues (e.g. a root), where they settle and encyst, in a rapid process that takes place within minutes. During this process, zoospores ventrally orient themselves towards the root surface as they encyst. Encystment is a rapid process that involves the detachment of the two flagella and secretion of glycoproteins and other molecules to form a primary cell wall. Cysts firmly attach to the root surface due to adhesive molecules (glycoproteins) situated on the newly formed cell wall. Encystment is induced by high concentrations of nutrients and other molecules present in root exudates or in a medium, such as isoflavones and phosphatidic acid, and also by high concentrations of exogenous Ca^{2+}, or by physical agitation or zoospore autoaggregation. The cyst germinates by developing a germ tube on its attached side towards the root surface. Normally, cysts begin to germinate within 30 minutes and germ tubes form in 2-3h. The germ tube can penetrate the host tissue directly or by formation of an appressorium via the anticlinal walls between the epidermal cells of the root. During this penetration process, the cytoplasm migrates from the cyst into the germ tube and to the appressorial vesicle which, by a combination of cell wall-degrading enzymes and mechanical pressure, is able to breach the plant epidermis. Aseptate hyphae grow and ramify through the living plant tissue developing a mesh of absortive mycelium, that acquires nutrients from host cells via haustoria. This mycelium eventually produce new sporangia by which the asexual stage may be repeated many times during infection of a plant, and thus the pathogen inoculum increases, along with its spreading and infection possibilities (Erwin and Ribeiro, 1996; Kamoun, 2000; Van West *et al.,* 2003; Judelson and Blanco, 2005).

In the sexual cycle, oosporogenesis generates oospores with a thick wall (of approximately 2 μm) adapted to harsh environmental conditions. This process involves the fecundation of the oogonium by the antheridium. After meiosis has occured in these structures, the antheridial nucleus passes into the oogonium and both nuclei fuse by means of karyogamy.

The oogonium expands and develops a single oospore, which can persist in the soil or in infected tissues in a dormant stage and then germinate under suitable conditions to produce a single or multiple germ tubes that can form mycelia and sporangia. These sporangia produces zoospores, thus allowing the asexual cycle to be repeated (Erwin and Ribeiro, 1996; Kamoun, 2000; Van West *et al.,* 2003; Judelson and Blanco, 2005).

P. cactorum-*host plant interaction and disease symptoms*

Pathogenicity comprises all the cellular and molecular processes that are necessary for the pathogen to establish and maintain a successful infection. The first stage of infection is recognition of the host by *P. cactorum*, and this involves different mechanisms of host targeting by zoospores. Zoospores can target a host tissue (e.g. root) by electrotaxis or chemotaxis. Electrotaxis is a mechanism by which zoospores are attracted to weak electric fields around plant roots formed by ionic currents (the higher the salt concentration, the weaker the electrical field), and it permits zoospores to distinguish living roots from dead ones as well as to select a suitable infection site (root tips, wounds, branch points). Chemotaxis is a mechanism that can be non-specific, such as chemotaxis towards ethanol and root exudates (amino acids), or specific towards some molecules in root exudates, such as isoflavones and valevaldehydes. Furthermore, chemotaxis can also be specific towards previously encysted zoospores on the root surface, a phenomenon known as autoattraction or autoaggregation that allows a higher probability of infection. Several molecules of the plant surface also induce zoospore encystment, for example cellulose, polyuronates and other components of root mucilage. Once the attached encysted zoospores germinate they must overcome the host barriers and resistance mechanisms to successfully penetrate and grow within it. Some pathogenicity factors of *Phytophthora* spp. include cell wall-degrading enzymes (endocellulases, pectinases, cutinases, β-1,3-glucanases...), production of toxins, formation of infection structures and detoxification of plant substances. At the same time, plant defense responses can be induced by some cell wall molecules and protein elicitors of *Phytophthora* spp. pathogens, e.g. the secreted acidic CAC-A elicitin from *P. cactorum* (Kamoun, 2000).

If the pathogen is able to overcome these plant defenses, infection and subsequent disease symptoms occur. *P. cactorum* mycelium grows intercellularly in infected tissues and forms haustoria that are special hyphal branches that penetrate living cells of the host for absortion of nutrients. Infected tissues become necrosed while the pathogen grows and colonizes other healthy plant tissues, mainly through the vascular system, which is also necrosed. The

proggressive necrosis of the vascular system obstructs the transport of water and nutrients from the roots to the rest of the plant, which gradually becomes chlorotic and wilted due to a collapse of the vascular system. In strawberry plants, for example, the infection and subsequent necrosis of roots, collar and crown cause symptoms similar to hydric stress. Plants in a later stage of the disease become weak and eventually die. Fruits that are in contact with *P. cactorum*-infested soil or water can also be infected and develop symptoms (e.g. leather rot of strawberry fruits). Disease caused by *P. cactorum* can be diagnosed by direct observation of plant symptoms and by observation of presence of oospores and other pathogen structures in infected tissues. (Erwin and Ribeiro, 1996; Tyler, 2002; Eikemo *et al*., 2003a; Van West *et al.,* 2003; Judelson and Blanco, 2005).

Phytophthora root rot of strawberry (*Fragaria x ananassa*)

The commercial strawberry (*Fragaria* × *ananassa* Duchesne), a hybrid of *F. chiloensis* (L.) Mill. and *F. virginiana* Mill., belongs to the Family Rosaceae. Strawberry is an important crop and its production has increased worldwide in the last years. The USA are the main strawberry producer, followed by Spain. In 2005, strawberry production in the USA was 1053280 metric tons (mt) and the area harvested was 21120 hectares (ha), whereas Spain produced 308000 mt of strawberries in a harvested area of 7600 ha, with a yield of 40526,3 kg/ha. Despite being the second producer, Spain is the main strawberry exporter in the world, exporting 226821 mt in 2004, 78.7% of its total strawberry production, and 42.4% of the total world exported strawberry (Food and Agriculture Organization (FAO), 2007. United Nations) (Figure 1.3). Approximately 94% of strawberry fruit in Spain is produced in Andalucia, mainly in the Huelva region, whereas 2% is produced in Catalonia, mostly in the Maresme region (Anuario Estadística Agraria, 2004. Ministerio de Agricultura, Pesca y Alimentación (MAPA), Spain. Revised in 2007).

Leather rot of strawberry fruit caused by *P. cactorum* is of economic importance to strawberry growers worldwide. Under favourable conditions for disease development, more than 80% of the strawberry fruit crop may become infected. Dark brown lesions occur on infected green fruit as the fruit ripens. These lesions later turn light brown with purple edges. These infected fruits rarelly fully ripen and they become tough with a rubbery or leathery texture. Their flavour and odour are also affected, becoming bitter and unpleasant (Figure 1.4).

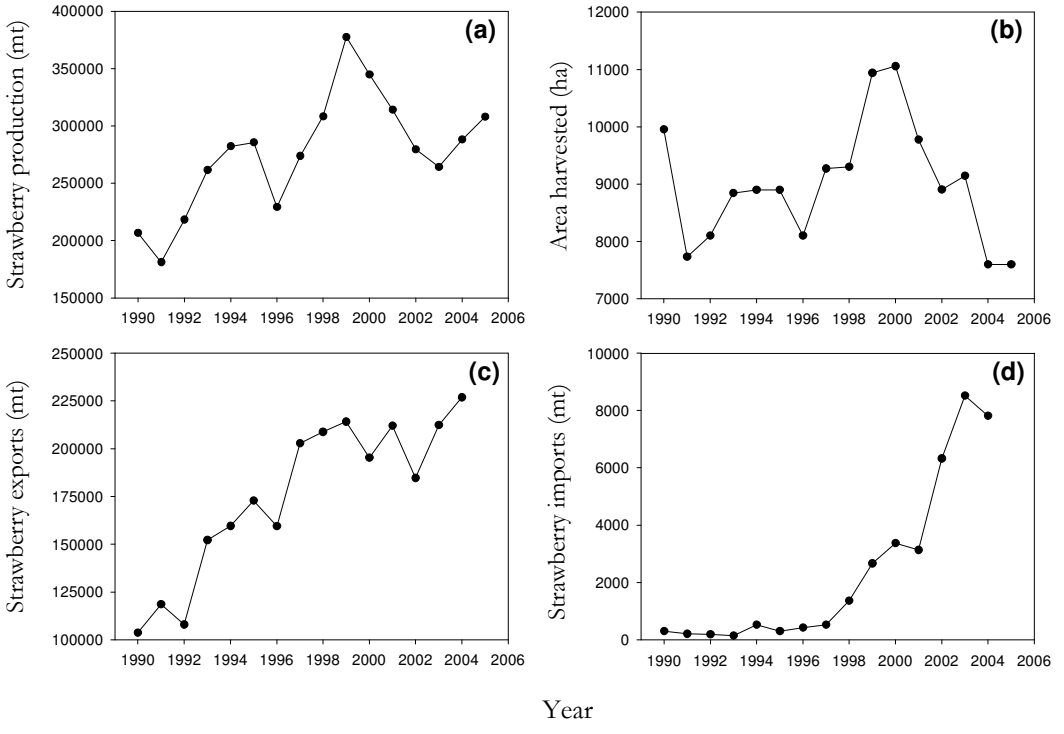

Figure 1.3. Evolution of the strawberry production (a), area harvested (b), and strawberry exports (c) and imports (d) in Spain from 1990 to 2004-2005. Source: Food and Agriculture Organization (FAO), 2007.

Crown, collar and root rot of strawberry plants caused by *P. cactorum* is commonly known as Phytophthora root rot and its typical symptoms are a wilt and dieback of the plant. First external symptoms are a change of colouration of young leaves that turn bluish green and wilt suddenly. These leaves then turn brown and the whole plant die within a few days. The first internal symptom of infection is a light-brown water-soaked appearance of the crown and collar tissues. Roots proggressively become necrosed and the crown becomes intensely and homogeneously brown. Leather fruit rot and Phytophthora root rot incidence increase with wetness duration when temperature is optimal (21-25ºC) and then decline. The initial infection by *P. cactorum* depends on the distribution of infected plants or infested soil. Water stress appears to be an essential factor that favours infection because the disease occurs mainly when the need of water is high, such as after transplantation and between flowering and harvesting (Erwin and Ribeiro, 1996; De los Santos *et al.*, 2003).

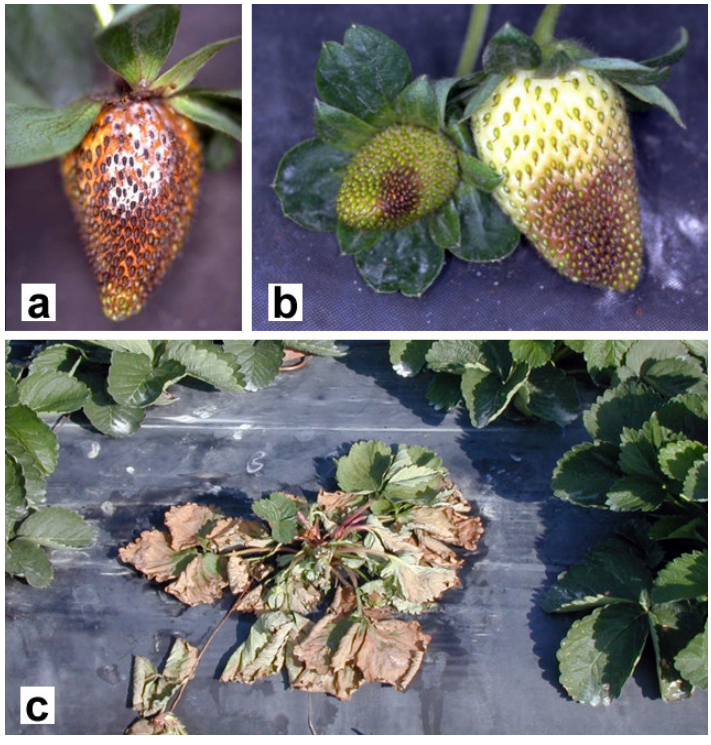

Figure 1.4. Disease symptoms of leather rot of fruits (a, b) and root rot of strawberry (c) caused by *P. cactorum*. Source: Anonimous.

The progressive nature of the infection and disease caused by *P. cactorum* can be interesting for practical purposes as it permits to establish a visual scale of different infection and disease levels that depends on differences of pathogen virulence or host susceptibility. By means of these different levels, infection and disease severity can be calculated, allowing statistical analysis and therefore the possibility to perform studies on the disease and the pathogen.

Disease control

Strawberry is a vegetatively propagated crop and all plant material used in commercial production fields is provided to the grower as bare rooted transplants. All leaves are removed from the plants after harvest from the nursery production fields and they are packed in plastic-lined boxes for shipment. These packed plants can be maintained between -4ºC and 4ºC. Just prior to transplantig, bare rooted transplants can be treated with a fungicide mixture to control pathogens that may be on the transplants or in the field soil (Martin and Bull, 2002).

Production of strawberry transplants in nurseries to be used in production fields is facing several important challenges on pathogen control since the prohibition of methyl bromide (MB) for soil fumigation. These plants must be produced in a manner that ensures control of soil-borne pathogens to minimize production loses in the nurseries. Furthermore, if the nursery stock provided to the grower is not clean, disease management in commercial production fields will be even more difficult. For example, in the USA, outbreaks of Phytophthora root rot in fields in the southeast were caused by transplants already infected with *P. cactorum* rather than from endemic populations of the pathogen (Huang *et al.*, 2004).

The commercial cultivars used are rather susceptible to different soil-borne pathogens and this results in yield decrease. Several strawberry diseases are caused by soil-borne pathogens that attack the collar and root of the plant. These soil-borne pathogens are mostly Oomycetes and fungi such as *Phytophthora* spp., *Pythium* spp., *Verticillium* spp., *Rhizoctonia* spp., and *Cylindrocarpon* spp.. There have been attempts to select strawberry cultivars resistant to different *Phytophthora* species such as *P. cactorum*, *P. citricola* and *P. fragariae*. The cv. Aromas is relatively tolerant to *P. cactorum* and *P. citricola*, whereas cvs. Diamante and Pajaro are more susceptible to these pathogens. Cultivars such as Camarosa and Diamante exhibited yield reductions of 45% to 60% when grown in non-fumigated soil relative to fumigated soil (Martin and Bull, 2002).

Normally, control of aerial pathogens is done by spraying leaves and fruits with chemical products, but control of soil-borne pathogens, and especially *Phytophthora* spp., is rather complex, and generally different control methods are applied combined to obtain better control results. The control of *P. cactorum* and other *Phytophthora* species is managed by preventive cultural methods such as soil disinfestation by solarization, use of organic mulches, improvement of soil drainage, removal of infected plant material, and by chemical control with protective fungicides (e.g. copper derivatives, dithiocarbamates, phtalimides) and by application of systemic fungicides to the soil (Fosetyl-Al and Metalaxyl) (Tuset, 2000). Currently, the only chemical products registered in Spain to control *Phytophthora* spp. are some formulations of Fosetyl-Al, Metalaxyl and Etridiazol (Ministerio de Agricultura, Pesca y Alimentación (MAPA), 2007).

Integration of biological control into control strategies for strawberry production is a plausible option, but it must be optimized to be efficient. There are some limitations of biological control in the strawberry production system. Disease control is rather complicated due to the dense root system of the strawberry plant and to several cycles of root growth and development that occur during the production season that would require repeated colonization

of the rhizosphere by the biocontrol agents. Although examples of biological control of *P. cactorum* in strawberry can be found in literature (Kurze *et al.*, 2001; Vestberg *et al.*, 2004; Porras *et al.*, 2007a; Porras *et al.*, 2007b), more research is needed in order to improve consistency of biocontrol of strawberry root disease and to understand the mechanisms of action of the biological control agents (BCA) involved in disease suppression. Biological control of *Phytophthora* spp. by different BCAs, alone or in combination with chemical treatments, has been reported in apple trees (Utkhede and Smith, 1993), raspberry (Valois *et al.*, 1996), *Citrus* sp. (Yang *et al.*, 1994; Steddom *et al.*, 2002), angelica trees (Okamoto *et al.*, 2000), cucumber (Lee *et al.*, 2003; Khan *et al.*, 2004), alfalfa (Silo-Suh *et al.*, 1994; Xiao *et al.*, 2002), soybean (Xiao *et al.*, 2002), tobacco (Cartwright and Spurr, 1998), bell pepper (Mercier and Manker, 2005), tomato (Lourenço Júnior *et al.*, 2006), and cocoa (Krauss and Soberanis, 2002; Krauss *et al.*, 2006). Variability of control efficacy is frequently high although combination of different BCAs or combination of BCAs and other control methods tend to reduce this variability and improve control efficacy and consistency.

Objectives

The main aim of the study in this chapter was the selection of *Pseudomonas fluorescens* and/or *Pantoea agglomerans* strains to be used in biological control of *Phytophthora cactorum* in strawberry and the determination of their efficacy in disease control when applied individually or in combination. A priori, a *P. cactorum*-strawberry model system was developed to perform plant-pathogen interaction studies as well as *ex vivo* and *in planta* screenings for biocontrol strains. Finally, the mechanisms of action of the most efficient biocontrol strains were determined.

Development and Evaluation of a Detached Leaf Inoculation Method for Screening of Biocontrol Agents against *Phytophthora cactorum* in Strawberry

Abstract

A detached leaf inoculation method was developed for screening biological control agents against *Phytophthora cactorum ex vivo* and was compared to *in planta* assays. Two pathogen inoculation methods (puncture and cut) were tested and proved to be equally effective to produce *P. cactorum* infection on strawberry leaves. Young leaves were significatively more susceptible to *P. cactorum* than mature and old leaves, and reached high infection severity levels 10 days after being inoculated with zoospore suspensions from 18-day-old *P. cactorum* cultures, while *P. cactorum*-inoculated strawberry plants required 45 days to clearly develop disease symptoms. This *ex vivo* method was also effective to evaluate biocontrol activity of bacterial strains against *P. cactorum*, and permitted to select strains that reduced infection more than 50%. Most of the strains tested behaved consistently *ex vivo* and *in planta*. This method allowed penetration and infection of leaf tissues by the pathogen, and colonization by bacterial cells, thus protecting leaf tissues from pathogen infection, as microscope observations showed.

Keywords: Phytophthora root rot, *Pseudomonas fluorescens*, *Pantoea agglomerans*, biological control agent, *ex vivo* assay.

Introduction

The Oomycete *Phytophthora cactorum* (Lebert & Cohn) J. Schröter is commonly found in temperate regions and infects more than 200 plant species, including several woody plants like *Prunus* sp., *Pyrus* sp. and *Malus* sp. (Tuset, 2000; Latorre *et al.*, 2001; Thomidis, 2003), ornamentals and other commercially valuable plants like strawberry, in which was first reported as the causal agent of root and crown rot in 1954 in Germany (Eikemo *et al.*, 2003a). On cultivated strawberry (*Fragaria x ananassa* Duchesne), *P. cactorum* causes collar, crown and root rot and leather rot of fruit. Infected plants show root necrosis and red-brown lesions within the crown, and subsequently, wilting of the plants. Under favourable environmental conditions, high humidity and poor soil drainage, sporangia and zoospores are produced in infected plant tissues and rapidly spread through running water. Motile biflagellate zoospores released from sporangia are attracted to a nearby host by chemotaxis. When they reach a leaf or root surface they encyst in response to chemical or physical stimulation, secrete adhesive substances that glues them to the host surface, and germinate, producing a germ tube that can penetrate and colonize the host plant (Erwin and Ribeiro, 1996; Hardham, 2001;Judelson and Roberts, 2002). Control of *P. cactorum* is based on cultural strategies and chemical treatments with soil disinfectants (1,3-dichloropropene, chloro-picrin, metham sodium and iodomethane), copper-based fungicides or Metalaxyl and Fosetyl-Al (Ellis *et al.*, 1998; Matheron and Porchas, 2000; De Liñán, 2003; Eikemo *et al.*, 2003a; Kuhajek *et al.*, 2003; De Cal *et al.*, 2004). This pathogen propagates by running water and its oospores and other resistant structures remain in soil and are difficult to eliminate. Consequently, preventive control

is more effective than treatments applied after the pathogen has attacked the plant and the disease has spread. Although biological control of *P. cactorum* has been reported (Kurze *et al.*, 2001), its practical application is still very far from chemical control and further investigation on screening methods is needed to find new biological control agents.

Whole plant assays have been widely used to assess *Phytophthora* spp. strain virulence (Thomidis *et al.*, 2002; Bhat *et al.*, 2006), plant resistance to *Phytophthora* spp. infection (Pazdernik *et al.*, 1997; Sowik *et al.*, 2001; Thomidis *et al.*, 2001; Eikemo *et al.*, 2003a; Eikemo *et al.*, 2003b), or screening for biological control agents against *Phytophthora* species (Valois *et al.*, 1996; Cartwright and Spurr, 1998; Shang *et al.*, 1999; Okamoto *et al.*, 2000; Kurze *et al.*, 2001; Steddom *et al.*, 2002; Xiao *et al.*, 2002; Vestberg *et al.*, 2004). However, whole plant assays are time- and space-consuming and disease symptoms caused by *P. cactorum* are well developed at least 30 to 45 days after inoculation. Detached organ inoculation methods could reduce disease incubation period and plant material size, and could be used under different conditions. So, it should be useful to develop *ex vivo* inoculation methods that permit to perform screening for biological control agents as well as evaluation of cultivar susceptibility, pathogen virulence and other plant-pathogen interaction studies.

Ex vivo and seedling inoculations have been used as rapid methods for determination of pathogen strain virulence (Downer *et al.*, 2001; Jia *et al.*, 2003; Hansen *et al.*, 2005; Morocko *et al.*, 2006), cultivar or host susceptibility (Dorrance and Inglis, 1997; Iwaro *et al.*, 1997; Thomidis *et al.*, 2001; Nyassé *et al.*, 2002; Thomidis *et al.*, 2002; Moragrega *et al.*, 2003; Denman *et al.*, 2005; Gevens *et al.*, 2006), to evaluate fungicide (Sedegui *et al.*, 1999) or peptide (Ali and Reddy, 2000) control efficacy, and to assess interaction between the pathogen and a biological control agent (Montesinos *et al.*, 1996; Elson *et al.*, 1997; Pusey, 1997; Lagopodi *et al.*, 2002; Bolwerk *et al.*, 2005) in different pathosystems, but not in the *P. cactorum*-strawberry pathosystem. Since *P. cactorum* is a soil-borne pathogen, natural infection occurs through the root system. Because of that, *ex vivo* methods using different detached aerial organs should be compared to root-inoculated whole plant assays.

The purpose of the present study was to develop a detached strawberry organ assay to be used for screening bacterial strains for *P. cactorum* biological control, as well as other plant-pathogen interaction studies. This *ex vivo* method was compared to whole plant assays. Additionally, effect of leaf age and *P. cactorum* strain virulence were evaluated by the developed detached leaf test. Infection process and ultrastructural changes on inoculated leaves were monitored by light and transmission electron microscopy.

Materials and Methods

Phytophthora cactorum strains

P. cactorum strains 170, 489, 490 and 642 isolated from strawberry lesions and provided by the Plant Health Laboratory (Huelva, Spain) were used in experiments. Sporangia and zoospores were obtained from 12- to 25-day-old cultures grown on modified V8 agar (12.5 g of tomato concentrate, 2.85 g of $CaCO_3$ and 16 g of agar per liter) (Dhingra and Sinclair, 1987) at 22 ± 1ºC under a 16-h light photoperiod (Sanyo, Tokyo, Japan). Zoospores of *P. cactorum* were collected by adding sterile distilled water in culture plates (approximately 5 ml in each) and scraping the culture surface with a Digralsky spreader. The suspension was collected with a micropipette and maintained at 5ºC for at least 3 h to induce sporangia maturation, followed by 1 h at room temperature to let sporangia release the zoospores. The concentration of zoospores was determined with an haemocytometer (Thoma, Brand, Germany) and adjusted to the desired dose.

Bacterial strains

Bacterial strains of *Pseudomonas fluorescens* and *Pantoea agglomerans* were obtained from the strain collection of the Institute of Food and Agricultural Technology (Girona, Spain) and selected for their putative biocontrol activities based upon preliminary laboratory assays. Additionally, four strains of *P. fluorescens* from culture collections reported in literature as biocontrol agents were included (Table 1).

Bacteria were stored at -80ºC in 20% glycerol Luria Bertani broth (LB) (Maniatis *et al.*, 1982), and were routinely cultured on LB agar at 23ºC prior to experiments. Bacterial suspensions were prepared from 24-h LB agar cultures grown at 23ºC, inoculated into 10 ml of LB broth in culture tubes and incubated at 23ºC on a rotatory shaker at 150 rpm for 24 h. Cells were then pelleted by centrifugation at 8000 x *g* for 15 min, resuspended in sterile distilled water and the final concentration was adjusted to 10^8-10^9 cfu ml^{-1}.

Table 1. Strains of *Pseudomonas fluorescens* and *Pantoea agglomerans* used in *ex vivo* and *in planta* screening for biocontrol of *Phytophthora cactorum*.

Bacterial strain	Species	Origin	Source[1]
EPS282	*P. fluorescens*	Alfalfa root	INTEA
EPS290	*P. fluorescens*	Maize root	INTEA
EPS328	*P. fluorescens*	Lettuce root	INTEA
EPS384	*P. fluorescens*	Weed leaf	INTEA
EPS435	*P. agglomerans*	Quince blossom	INTEA
EPS454	*P. agglomerans*	Peach flower	INTEA
EPS458	*P. agglomerans*	Apple blossom	INTEA
EPS475	*P. agglomerans*	Peach blossom	INTEA
EPS514	*P. agglomerans*	Pear blossom	INTEA
EPS539	*P. fluorescens*	Pear blossom	INTEA
EPS560	*P. agglomerans*	Hawthorn leaf	INTEA
EPS599	*P. fluorescens*	Hawthorn flower	INTEA
EPS894	*P. fluorescens*	Apple leaf	INTEA
BL915	*P. fluorescens*	Cotton root	S. Hill
Ps15	*P. fluorescens*	Sugarcane root	M. N. Nielsen
Q4-87	*P. fluorescens*	Wheat root	L. Thomashow
SBW25	*P. fluorescens*	Wheat root	L. Thomashow

[1]INTEA, Institute of Food and Agricultural Technology, University of Girona, Spain; Steve Hill, Syngenta Biotechnology, Inc. Research Triangle Park, NC, USA; Mette Neiendam Nielsen, Department of Ecology and Molecular Biology. Section of Genetics and Microbiology, Royal Veterinary and Agricultural University, Denmark; Linda Thomashow, US Department of Agricultural Research Service, Root Disease and Biological control Research Unit, Washington State University, USA.

Plant material and maintenance

Cold-stored strawberry plants of cultivars Camarosa and Diamante were used in all experiments. Plants had been maintained at approximately 2ºC for at least two months when delivered from the grower. Plants used to obtain leaves were planted in 500-ml pots in peat:perlite (3:1) substrate and maintained in greenhouse at 25 ± 4ºC with a 16-h photoperiod for two months.

Infectivity of *P. cactorum* strains on detached strawberry organs

The infectivity of zoospore suspensions from cultures of different maturity stages (18 and 25 day-old) of four *P. cactorum* strains (170, 489, 490 and 642) was tested in two different detached strawberry organs: leaves and petioles. The experiment was repeated twice in two different strawberry cultivars. Young leaves and their petioles were collected from strawberry plants of cultivars Camarosa and Diamante. Leaves were excised from the petiole and leaflets were separated. 2-cm long petiole fragments were cut. Leaflets and petioles were surface-disinfected by immersion in a diluted solution of sodium hypochlorite (2% active chlorine) for 1 min, rinsed three times with sterile distilled water and placed under an air stream to remove excess water. Each leaflet was wounded with a scalpel on the reverse of the midvein, whereas each petiole was longitudinally cut with a scalpel. Organs were inoculated with a 20-µl drop of a pathogen suspension ($1x10^4$-$5x10^4$ zoospores per ml) obtained from 18- or 25-day-old cultures. Controls were inoculated with 20 µl of sterile distilled water. Inoculated organs were placed on wet sterile filter paper into 43 x 33 x 8 cm transparent plastic boxes, and incubated at 22 ± 1ºC and a 16-h light photoperiod in a controlled environment chamber (model PGR-15, Conviron Winnipeg, MB, Canada). Three sets of three organs per treatment were arranged in a randomized experimental design. Infection levels were assessed five and 10 days after inoculation according to the following scale (Figure 1): 0= no infection, 1= up to 1/3 of the organ infected, 2= up to 2/3 of the organ infected, and 3= up to 3/3 of the organ infected. Infection severity (*S*) was calculated using the following formula:

$$S = \frac{\sum_{i=1}^{n} I_i}{n \cdot 3} \cdot 100$$

where *S* is the infection severity per repetition, I_i is the corresponding severity index per inoculation and *n* is the number of inoculated organs per set.

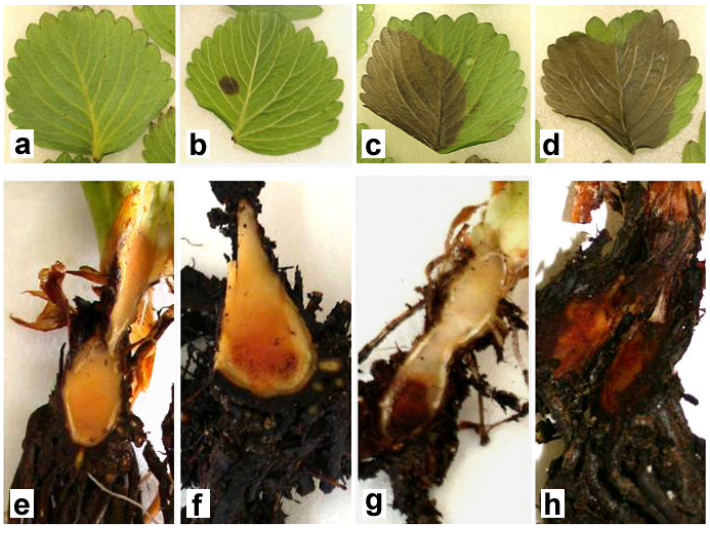

Figure 1. Necrosis levels established to measure infection caused by *P. cactorum* in strawberry leaves and collar roots. a-d: Infection levels (0 to 3) on strawberry leaflets. e-h: infection levels (0 to 3) in the crown and collar of strawberry plants.

Effect of strawberry leaf age and inoculation method on *P. cactorum* infection on detached leaves

Strawberry leaves of different maturity stages (young, mature and old leaves) were collected from strawberry plants of cultivars Camarosa and Diamante. Leaves were excised from the petiole and leaflets were separated. Leaflets were surface disinfected as described above. Two types of wounds were made on the reverse of each leaflet: a puncture on the limbus with a sterile needle and a cut on the midvein with a scalpel. A 20-µl drop of *P. cactorum* 489 suspension ($5x10^4$ zoospores per ml) was inoculated onto the wounds made on each leaflet. Controls were inoculated with 20 µl of sterile distilled water. Inoculated leaflets were placed with the abaxial face up on wet sterile filter paper into transparent plastic boxes, and incubated at 22 ± 1ºC with a 16-h light photoperiod in a controlled environment chamber. Infection levels were assessed five and 10 days after inoculation as described above. Infection severity (*S*) was calculated using the formula previously described. Five sets of three leaflets per treatment were arranged in a randomized experimental design and the experiment was conducted twice. Experiment 1 was performed with leaves of cvs. Camarosa and Diamante and experiment 2 was performed only with leaves of cv. Diamante.

Infectivity of *P. cactorum* in strawberry plants

Strawberry plants of cultivars Camarosa and Diamante were inoculated with zoospore suspensions of *P. cactorum* strains 170 and 489 obtained from three different maturity stage cultures (12-, 18- and 25-day-old cultures). Concentration of zoospore suspensions ranged from $5x10^3$ to $5x10^4$ zoospores per ml. Plants were inoculated by irrigation with 20 ml of zoospore suspension and incubated for 45 days at 22 ± 1ºC and a 16-h light photoperiod in a controlled environment chamber (model PGR-15, Conviron Winnipeg, MB, Canada). Control plants were irrigated with 20 ml of distilled water. To favour infection by the pathogen, plants were kept in transparent plastic bags for 48 h after inoculation. Holes were made in the plastic bags to avoid excessive CO_2 accumulation. Disease levels were assessed 45 days after inoculation by observing the necrosis progression inside the collar root and according to the following scale (Figure 1): 0= no necrosis, 1= up to 1/3 of the collar necrosed, 2= up to 2/3 of the collar necrosed, and 3= up to 3/3 of the collar necrosed. Three sets of two plants per treatment were arranged in a randomized experimental design. Disease severity was calculated using the formula described above for detached organ assays, where S is the disease severity per repetition, I_i is the necrosis index per plant and n is the number of plants per set.

Ex vivo* and *in planta* screening of bacteria for biological control of *P. cactorum

1. *Ex vivo* assay

Biocontrol activity of 17 *P. fluorescens* and *P. agglomerans* strains (Table 1) against *P. cactorum* infection was tested. Young leaves of strawberry plants of cultivars Camarosa and Diamante were excised from the petiole and leaflets were separated, surface disinfected as described above and placed under an air stream to remove excess water. Two wounds were made with a sterile needle on the reverse of the limbus of each leaflet. Wounded leaflets were treated by immersion in a 10^8 cfu ml^{-1} suspension of the bacterial strain and placed under an air stream. A 20-µl drop of *P. cactorum* 489 suspension ($5x10^4$ zoospores per ml) was inoculated onto each wound made on each leaflet. Controls consisted of non-bacteria-treated leaflets inoculated with the pathogen, and bacteria-treated leaflets inoculated with 20 µl of sterile distilled water. Inoculated leaflets were placed with the abaxial face up on wet sterile

filter paper into transparent plastic boxes and incubated at 22 ± 1ºC and a 16-h light photoperiod in a controlled environment chamber. Infection levels were assessed five, 10 and 15 days after inoculation, and infection severity was calculated as described above. Two independent experiments were performed. Experiment 1 was performed on cv. Diamante leaves and experiment 2 on cv. Camarosa leaves. In both experiments, each treatment consisted of three sets of six leaflets, which were arranged in a randomized experimental design.

2. *In planta* assay

Two independent experiments were conducted on cv. Diamante strawberry plants using two different types of *P. cactorum* inoculum and inoculation methods. In both experiments, a total of five bacteria applications were performed from planting the strawberry plants to 28 days after *P. cactorum* inoculation. *P. fluorescens* EPS328, EPS599, EPS894, BL915, Ps15, Q4-87 and SBW25 were tested (Table 1). First bacteria application was performed at the moment of planting by dipping each plant root system in a 10^9 cfu ml^{-1} bacterial suspension. Treated plants were introduced into 500-ml pots containing a peat:perlite (3:1) mixture and maintained in a greenhouse at 25 ± 5ºC and a 16-h photoperiod during the experiments. Two additional bacterial treatments were performed on strawberry plants seven and 14 days after first treatment and before *P. cactorum* inoculation by irrigation with 8 ml of 10^9 cfu ml^{-1} suspensions. Non-treated control plants were irrigated with distilled water.

28 days after planting, *P. cactorum* was inoculated on potted plants. In first experiment, each plant was inoculated by irrigation with 20 ml of *P. cactorum* 489 suspension ($2x10^5$ zoospores per ml). In second experiment, each plant was inoculated with the pathogen by placing 40 ml (approximately $2x10^6$ propagules per plant) of a pathogen-infested substrate around the roots. To prepare the *P. cactorum*-infested substrate, mycelium plugs of *P. cactorum* 489 from a 15-day-old culture on modified V8 agar were placed in a sterilized 10-liter mixture of wheat bran:vermiculite (1:1) containing 600 g of soybean flour and 1700 ml of distilled water. The inoculated substrate was incubated at 22 ± 1ºC and a 16-h light photoperiod for 28 days (Kurze *et al.*, 2001), and reached approximately $5x10^4$ propagules per ml of inoculum. To favour infection by the pathogen, plants were kept in transparent plastic bags for 24 h to reach high relative humidity. Holes were made in the plastic bags to avoid excessive CO_2 accumulation. Two additional bacterial treatments were performed 14 and 28 days after pathogen inoculation by irrigation with bacterial suspensions. Plants were maintained in a greenhouse at 25 ± 5ºC, with a 16-h photoperiod. 45 days after pathogen inoculation, levels of necrosis of collar and crown root were assessed as described above. In first experiment, two sets of five plants per treatment were arranged in a randomized experimental design, whereas in second experiment, three sets of five plants per treatment were used.

Plant tissue processing for light and transmission electron microscopy (TEM)

Young strawberry detached leaves were surface disinfected, cut onto the midvein, treated by immersion in a 10^8 cfu ml^{-1} suspension of *P. fluorescens* EPS894 and inoculated with a 20-µl drop of a *P. cactorum* 489 suspension at $5x10^4$ zoospores per ml, as described in previous assays. Positive non-treated controls consisted of leaves inoculated only with *P. cactorum*. Non-bacteria-treated and non-pathogen-inoculated leaves were included as negative controls. Three and six days after pathogen inoculation, a 5-mm^2 area around the inoculation point was cut into pieces of approximately 1 mm^2 and immediately fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 6 h at 4ºC with gentle shaking. All samples were rinsed twice in 0.1 M sodium cacodylate

buffer at 4ºC and post-fixed for 12 h in 1% osmium tetroxide in the same buffer at 4ºC, dehydrated in a graded series of acetone, and embedded in low viscosity 'Spurr' resin (Spurr, 1969). Semi-thin sections (1.0 μm) were cut from the resin-embedded material with glass knives. Sections were mounted on glass slides and contrasted with methylene blue. Observations were performed under a Leica DMR-XA optic microscope (Leica Microsystems, GmbH, Germany). Ultra-thin sections (0.1 μm) were collected on cupper grids (G2200C, AGAR Scientific Ltd., Stansted, England) and stained with lead citrate (Reynolds, 1963). Observations of ultra-thin sections were performed with a Zeiss EM910 transmission electron microscope (TEM) (Carl Zeiss Inc., Germany) at 60 kV.

Data analysis

Significance of the effect of treatments was tested by analysis of variance (ANOVA). Means were separated using the Fisher's protected least significant difference (LSD) test or the Tukey's studentized range (HSD) test at $P \leq 0.05$. The analysis was performed with the GLM procedure of the PC-SAS software (version 8.2, SAS Institute, Cary, NC, USA). Homogeneity of variances was determined by Barlett's test.

Results

Infectivity of *P. cactorum* strains on detached strawberry organs

Symptoms of *P. cactorum* infection after artificial inoculation of detached strawberry leaves and petioles began to be evident three to five days after inoculation. Pathogen strains produced progressive necrosis that expanded from the inoculation point through the leaf or the petiole. Maximal severity values were obtained 10 days after inoculation of zoospore suspensions from 18-day-old *P. cactorum* cultures. Infection of petioles affected the vascular tissue, which quickly collapsed and became necrotic, whereas infection progress in leaflets was slower and intermediate severity levels could be observed, depending on the leaflet area affected. Organs inoculated with sterile distilled water did not develop infection symptoms.

Infection severity was assessed five and 10 days after inoculation of zoospore suspensions from 18- and 25-day-old cultures on detached strawberry organs. In first experiment (cv. Camarosa), suspensions from 18-day-old cultures produced higher severity levels (up to 66.7%) than suspensions from 25-day-old cultures (up to 48.2%), but in second experiment (cv. Diamante) suspensions from both culture maturity stages produced similar infection levels (up to 66.7%) in both leaves and petioles.

Statistical analysis was performed with severity data collected 10 days after inoculation of zoospore suspensions from 18-day-old *P. cactorum* cultures. No significant differences on severity values were observed between the two independent experiments ($P=0.6606$) although different strawberry cultivars were used. The four *P. cactorum* strains were able to infect and develop symptoms when artificially inoculated on detached strawberry organs. All strains were similarly virulent, since no differences were observed among them ($P=0.0774$), and both organs (leaf and petiole) were similarly susceptible to *P. cactorum* infection ($P=0.2456$). However, a slight significant effect of *P. cactorum* strain-strawberry organ interaction was observed ($P=0.0461$). The virulence of each strain on strawberry detached organs was consistent in both experiment replicates (Figure 2). Infection severity on leaves ranged from 30-40% (strains 489 and 642) to 60-70% (strains 170 and 490), whereas on petiole strains 170, 489 and 642 produced higher severity levels (50-70%) in both experiments, while strain 490 produced lower severity (20-30%) in experiment 1. Although global analysis of

variance indicated no differences among strains, Fisher's unprotected least significance test indicated that strains 170 and 490 were significantly more virulent than strains 489 and 642 on strawberry leaves (Figure 2).

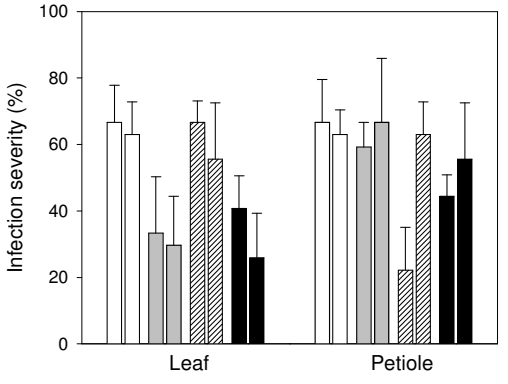

Figure 2. Infection severity on detached strawberry organs 10 days after inoculation with *P. cactorum* strains. Zoospore suspensions from 18-day-old cultures of strains 170 (white bars), 489 (grey bars), 490 (dashed bars) and 642 (black bars) were used. Values are the mean of three sets of three leaflets or petiole fragments. Error bars represent the mean standard error. Experiments 1 and 2 are represented by two consecutive bars of the same colour.

Effect of strawberry leaf age and inoculation method on *P. cactorum* infection on detached leaves

Artificial inoculation of *P. cactorum* 489 zoospore suspensions on wounded detached strawberry leaves resulted in infection and tissue colonization. Symptoms developed progressively from the wound through the leaf. Five days after inoculation, necrosis was observed surrounding the limbus puncture or expanding along the midvein from the cut, depending on the inoculation method used. Wounds performed by puncture on the limbus were smaller than midvein cuts and necrosis expanded through the leaf surface, whereas in midvein wounds necrosis expanded not only through the leaf surface but also along the vascular tissues, affecting secondary veins as well as the midvein. Maximal severity values were obtained 10 days after inoculation in all treatments. Leaves inoculated with sterile distilled water did not show infection symptoms.

Since no significant effect of strawberry cultivar was observed in experiment 1 (P=0.1674), in which leaves from 'Camarosa' and 'Diamante' strawberry plants were used, pooled data from both cultivars were used in further statistical analysis.

The ANOVA indicated a significant effect of leaf age in infection severity 10 days after inoculation in both experiment replicates (P<0.0001). Young leaves were significantly more susceptible to the infection, reaching high severity values (up to 93% in experiment 1 and 60-80% in experiment 2) (Figure 3). Susceptibility of mature and old leaves to *P. cactorum* infection was similar and significantly lower than young ones, with severity values ranging from 10% to 55%, depending on the inoculation method and the experiment. Moreover, disease progress and symptom development in young strawberry leaves was faster than in older ones, since five days after inoculation infection severity was moderate (30-40%, for inoculations on limbus puncture) or high (50-70%, for inoculations on midvein cut).

No significant differences of infection severity between inoculation methods were observed in young leaves in both experiments (P=0.6472, and P=0.6436, respectively). Only significant differences between methods were observed in mature and old leaves in experiment 1 (P<0.0001 and P=0.0339, respectively), where infection severity was 20% higher in old leaves inoculated by a cut on the midvein.

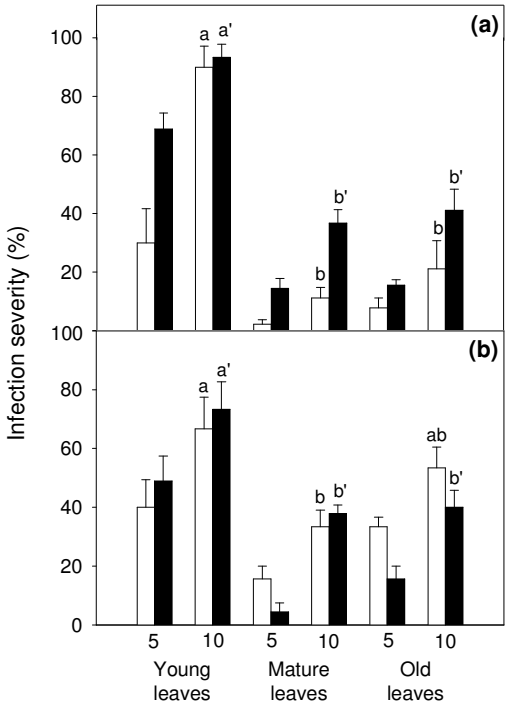

Figure 3. Effect of strawberry leaf age and inoculation method on *P. cactorum* infection severity. Infection severity was assessed five and 10 days after inoculation of *P. cactorum* 489 zoospore suspensions on young, mature and old detached strawberry leaves of cv. Camarosa and Diamante (a: experiment 1) or cv. Diamante (b: experiment 2). A 20-µl drop of zoospore suspension was placed on a puncture on the limbus (white bars) or a cut on the midvein (black bars) of each leaflet. Values are the mean of five sets of three leaflets per cultivar. Error bars represent the mean standard error. Bars headed by different letters are significantly different ($P \leq 0.05$) according to Tukey's least significance test.

Infectivity of *P. cactorum* in strawberry plants

P. cactorum infection on strawberry plants produced characteristic disease symptoms that were clear and well developed 45 days after pathogen inoculation. Highly affected plants showed internal collar and crown root brown necrosis, as well as a general wilting and progressive plant death.

Disease severity depended on strawberry cultivar and inoculum maturity stage ($P<0.0001$ and $P=0.0005$, respectively). The ANOVA also indicated a significant effect of cultivar-inoculum maturity interaction ($P<0.0001$). No significant effect of pathogen strain on disease severity was observed ($P=0.1889$), neither of its interaction with cultivar and pathogen maturity stage, nor the triple interaction ($P=0.3206$, $P=0.3631$, and $P=0.5568$, respectively).

'Camarosa' strawberry plants were poorly affected by pathogen infection and disease levels were low for all pathogen isolates and inoculum maturity stages. On the other hand, disease symptoms in 'Diamante' strawberry plants were clear and well developed 45 days after inoculation of zoospore suspensions from 18- and 25-day-old cultures. Inoculum infectivity in 'Diamante' plants increased linearly with pathogen culture age, since low disease severity levels were obtained in 12-day-old cultures, moderate in 18-day-old cultures and high in 25-day-old cultures (Table 2). Mortality was recorded through all the incubation time as percentage of dead plants. No dead plants of cv. Camarosa were observed, while in cv. Diamante up to 16.7% of plants died when inoculated with zoospore suspensions from 18-day-old cultures of *P. cactorum* strain 170, and 50% when inoculated with suspensions from 25-day-old cultures of *P. cactorum* strain 489.

Table 2. Disease severity (%) in 'Camarosa' and 'Diamante' strawberry plants 45 days after inoculation with zoospore suspensions of two *P. cactorum* strains from different culture maturity stages.

Strawberry cultivar	*P. cactorum* strain	Inoculum culture maturity stage[y]		
		12 day-old[z]	18 day-old	25 day-old
Camarosa (exp. 1)	170	11.1 (11.1)	0.0 (0.0)	5.6 (5.6)
	489	5.6 (5.6)	16.7 (16.7)	0.0 (0.0)
Diamante (exp. 2)	170	5.6 (0.0)	22.2 (14.7)	61.1 (5.6)
	489	5.6 (5.6)	38.9 (14.7)	83.3 (9.6)

[y] Plants were inoculated by irrigation with 20 ml of a zoospore suspension ($5x10^3$-$5x10^4$ zoospores per ml) obtained from *P. cactorum* cultures on modified V8 agar of different maturity stages.
[z] Severity values are the mean of three sets of two plants. Standard errors are presented into brackets.
Linear regression equations between disease severity and inoculum maturity stage were calculated in 'Diamante' for *P. cactorum* strain 170: $y=4.31x - 49.37$, $R^2=0.97$, and for *P. cactorum* strain 489: $y=5.99x - 67.28$, $R^2=0.99$, being (y) the disease severity and (x) the inoculum maturity.

Ex vivo and *in planta* screening of bacteria for biological control of *P. cactorum*

Symptoms of *P. cactorum* infection in leaves and plants corresponded to those described above in previous experiments. Pathogen infection produced progressive necrosis from the inoculation point through the leaf. Symptoms in plants affected the collar and crown root internally. Intensity of leaf and plant infection varied depending on bacterial treatment. Infection severity by *P. cactorum* in non-bacteria-treated detached leaves was 68.5% and 61.1% in experiment 1 and 2, respectively; whereas in *in planta* assays, disease severity in pathogen-inoculated strawberry control plants was lower (25-26%) (Table 3).

Treatment of strawberry leaves or plants with *P. fluorescens* or *P. agglomerans* strains resulted in none, moderate or high reduction of *P. cactorum* infection.

The detached leaf inoculation method permitted to evaluate biocontrol activity of bacterial strains against *P. cactorum* since different severity levels were obtained depending on the bacterial strain (Table 3). The global ANOVA of leaf infection severity values indicated a significant effect of the experiment (P=0.0128) and bacterial treatment (P<0.0001), but no significant effect of the experiment-bacterial treatment interaction (P=0.8210). Although differences in severity levels were observed in both *ex vivo* experiment replicates, efficacy in infection reduction was rather consistent for each strain. Additionally, strains selected from the *ex vivo* assay for evaluation *in planta* generally behaved in a similar way in disease control. The global ANOVA of the *in planta* assay indicated a significant effect of bacterial treatment (P<0.0001), but no significant effect of experiment and experiment-bacterial treatment interaction (P=0.6230 and P=0.3448, respectively).

Fisher's LSD test (showed in Table 3) permitted to group bacterial strains according to its efficacy in *P. cactorum* infection reduction. Strains *P. fluorescens* EPS328 and EPS384, and *P. agglomerans* EPS435 did not reduce *P. cactorum* infection in leaves, with infection severity values similar to those of *P. cactorum*-inoculated control leaves (severity > 45%). Strains *P. fluorescens* EPS282, EPS290, EPS539, BL915, Ps15 and Q4-87, and *P.*

agglomerans EPS514 and EPS560 showed a moderate control of *P. cactorum* infection in strawberry leaves, with severity values generally ranging from 20% to 45%, lower than those of non-bacteria-treated *P. cactorum*-inoculated leaves. Strains *P. fluorescens* EPS599, EPS894 and SBW25, and *P. agglomerans* EPS454, EPS458 and EPS475, reduced infection severity 70% or more, and severity values were lower than 20%. Some of these strains, especially *P. fluorescens* EPS599, EPS894 and SBW25, also reduced disease severity *in planta* (Table 3). Plants treated with *P. fluorescens* BL915 reached disease severity levels 15% higher than non-treated plants.

Table 3. Efficacy of *P. fluorescens* and *P. agglomerans* strains in biocontrol of *P. cactorum* in strawberry determined by *ex vivo* and *in planta* assays.

Bacterial strain[z]	Disease severity (%)[x]			
	Ex vivo[y]		*In planta*	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Non-treated	68.5	61.1	25.3	26.7
Pf EPS384	63.0	53.7	-	-
Pa EPS435	59.3	48.1	-	-
Pf EPS328	55.6	1.9	16.7	15.6
Pf BL915	31.5	13.0	40.0	42.2
Pf EPS282	29.6	20.4	-	-
Pf Ps15	29.6	18.5	13.3	6.7
Pf Q4-87	29.6	7.4	23.3	33.3
Pa EPS560	27.8	25.9	-	-
Pf EPS290	24.1	18.5	-	-
Pa EPS514	24.1	40.7	-	-
Pf EPS539	20.4	13.0	-	-
Pf EPS599	18.5	9.3	0.0	0.0
Pf EPS894	16.7	1.9	3.3	4.4
Pf SBW25	16.7	0.0	16.7	0.0
Pa EPS454	13.0	5.6	-	-
Pa EPS458	13.0	1.9	-	-
Pa EPS475	13.0	24.1	-	-
$LSD_{0.05}$	34.7	34.4	16.4	13.4
Mean	30.8	18.4	18.6	16.5

[x] Severity was assessed 15 days after leaf inoculation or 45 days after plant inoculation. Severity values are the mean of three sets of six detached leaflets in the *ex vivo* assay, or two sets of five plants (experiment 1) and three sets of five plants (experiment 2) in the *in planta* assay.

[y] *Ex vivo* experiment 1 was performed on cv. Diamante leaves while experiment 2 was performed on cv. Camarosa. Both *in planta* experiments were performed on cv. Diamante plants.

[z] Detached leaflets were dipped into 10^8 cfu ml^{-1} bacterial suspensions before pathogen inoculation in *ex vivo* assay. Plants were first treated by dipping their roots in 10^9 cfu ml^{-1} bacterial suspensions before planting. Additionally, four more treatments by irrigation with 8 ml of 10^9 cfu ml^{-1} bacterial suspensions were performed on plants before and after pathogen inoculation.

Light and transmission electron microscopy observations

Light microscopy observations of non-bacteria-treated strawberry leaves and leaves treated with *P. fluorescens* EPS894 and inoculated with *P. cactorum* are presented in Figure 4. No symptoms were observed in non-bacteria-treated and non-pathogen-inoculated leaves (Figure 4 a). Three days after inoculation of *P. cactorum*, leaf chlorenchyma appeared plasmolysed and disorganized (Figure 4 f) and *P. cactorum* hyphae were observed in the vascular tissue, near or inside xylem vessels (Figure 4 e), and six days after pathogen inoculation, a high deterioration of chlorenchyma and parenchyma cells was observed (Figure 4 b). On the other hand, no plasmolysis was observed in parenchyma and chlorenchyma cells of leaves treated with *P. fluorescens* EPS894 six days after inoculation of the pathogen (Figure 4 c). In a higher magnification, *P. cactorum* hyphae could be observed in vascular tissue, however, leaf cells and tissues did not appear as deteriorated as in non-bacteria-treated inoculated leaves (Figure 4 d). Additionally, high quantities of bacteria could be observed in xylem vessels and in broken parenchyma cells. These tissue preparations were subsequently prepared for and observed under a transmission electron microscope to corroborate light microscopy observations.

Transmission electron microscopy (TEM) observations of non-bacteria-treated and *P. fluorescens* EPS894-treated strawberry leaves inoculated with *P. cactorum* are also presented (Figures 5 and 6, respectively). All cells of non-pathogen-inoculated strawberry leaves presented a normal ultrastructure and organelles could be well differentiated, especially in chlorenchyma and parenchyma cells. No plasmolysis was observed in leaf tissues (Figure 5 a). However, three days after *P. cactorum* inoculation, leaves showed alterations in cell ultrastructure. Infected parenchyma and chlorenchyma cells presented electro-dense and irregularly-shaped chloroplasts and other organelles. Plasmolysis of parenchyma cells was also observed (Figure 5 b). Pathogen hyphal growth was observed in intercellular spaces and in vascular tissue three days after inoculation, although this hyphal growth was more evident six days after inoculation (Figure 5 c). Mature hyphae grew not only through the intercellular spaces and the vascular tissue but they were also observed attached to chlorenchyma and parenchyma cells and penetrating them by formation of haustoria (Figure 5 d, e and f). These cells presented an even more altered ultrastructure and severe plasmolysis.

When leaves were treated with *P. fluorescens* EPS894 previously to *P. cactorum* inoculation, alterations in cell ultrastructure were less important than those observed in non-bacteria-treated *P. cactorum*-inoculated leaves. Six days after bacterial treatment and pathogen inoculation, a high concentration of bacteria was observed inside xylem vessels and neighbouring cells near the inoculation point (Figure 6 a). Damaged or broken plant cells also contained bacteria (Figure 6 c and d). *P.cactorum* hyphae and bacteria were also observed together in xylem vessels (Figure 6 b) and in intercellular spaces (Figure 6 e and f). Bacterial cells and pathogen hyphae did not appear to interact directly, although a close contact between bacteria and *P. cactorum* was observed (Figure 6 f).

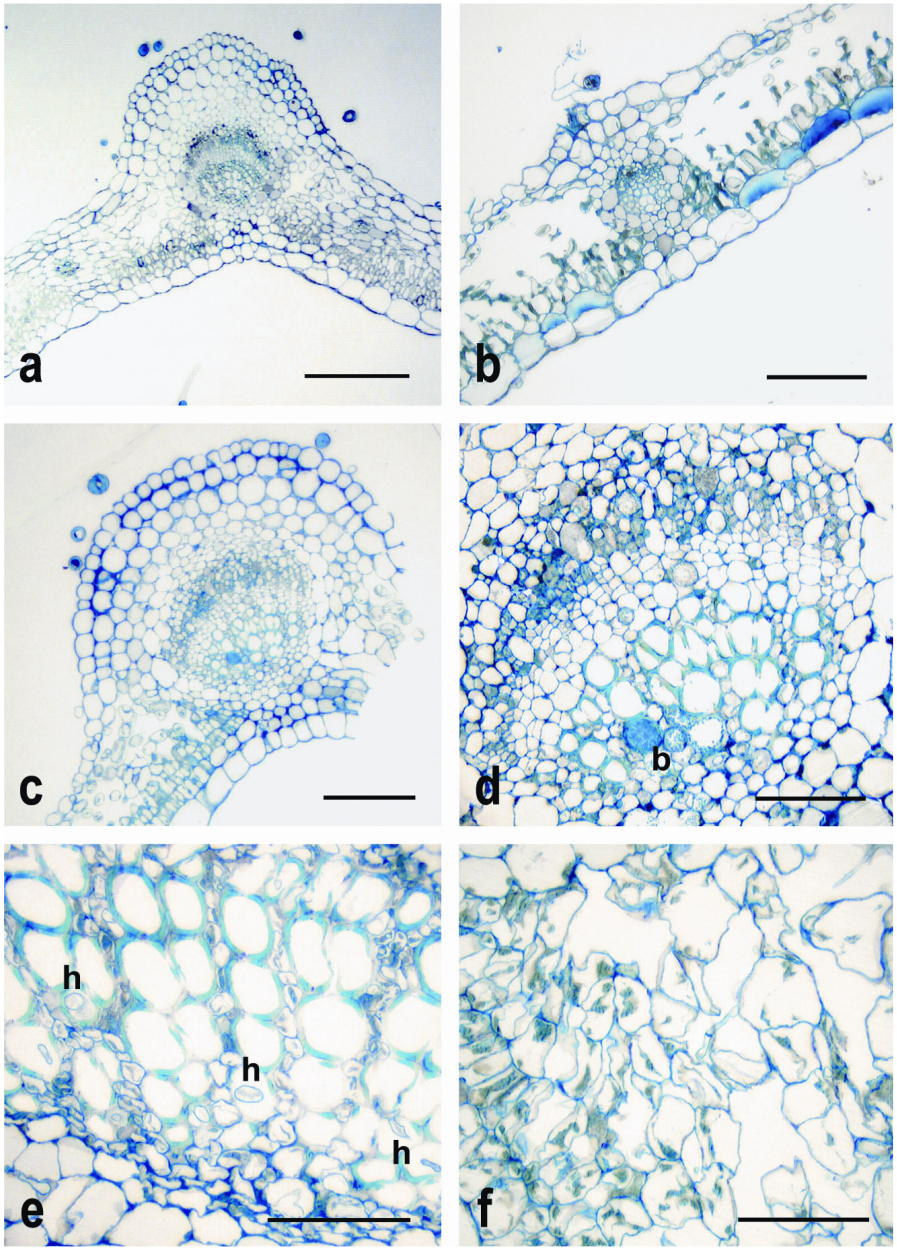

Figure 4. Light microscopy observations of the infection progression in young strawberry leaves inoculated with *Phytophthora cactorum*. (a) Non-bacteria-treated healthy leaf (scale bar: 200 µm). (b) Section of the infection margin of a leaf six days after inoculation of *P. cactorum* showing chlorenchyma degeneration (scale bar: 100 µm). (c, d) Inoculation point of a *Pseudomonas fluorescens* EPS894-treated leaf six days after *P. cactorum* inoculation, with no visible cell deterioration (c) (scale bar: 100 µm), and bacteria clearly observed inside xylem vessels and adjacent parenchyma cells (d) (scale bar: 50 µm). (e, f) Detail of vascular tissue and chlorenchyma cells, respectively, of a non-bacteria-treated leaf three days after *P. cactorum* inoculation showing a progressive deterioration of the cytoplasm, plasmolysis and presence of hyphae inside the xylem vessels (scale bars: 50 µm). b, bacteria; h, hypha.

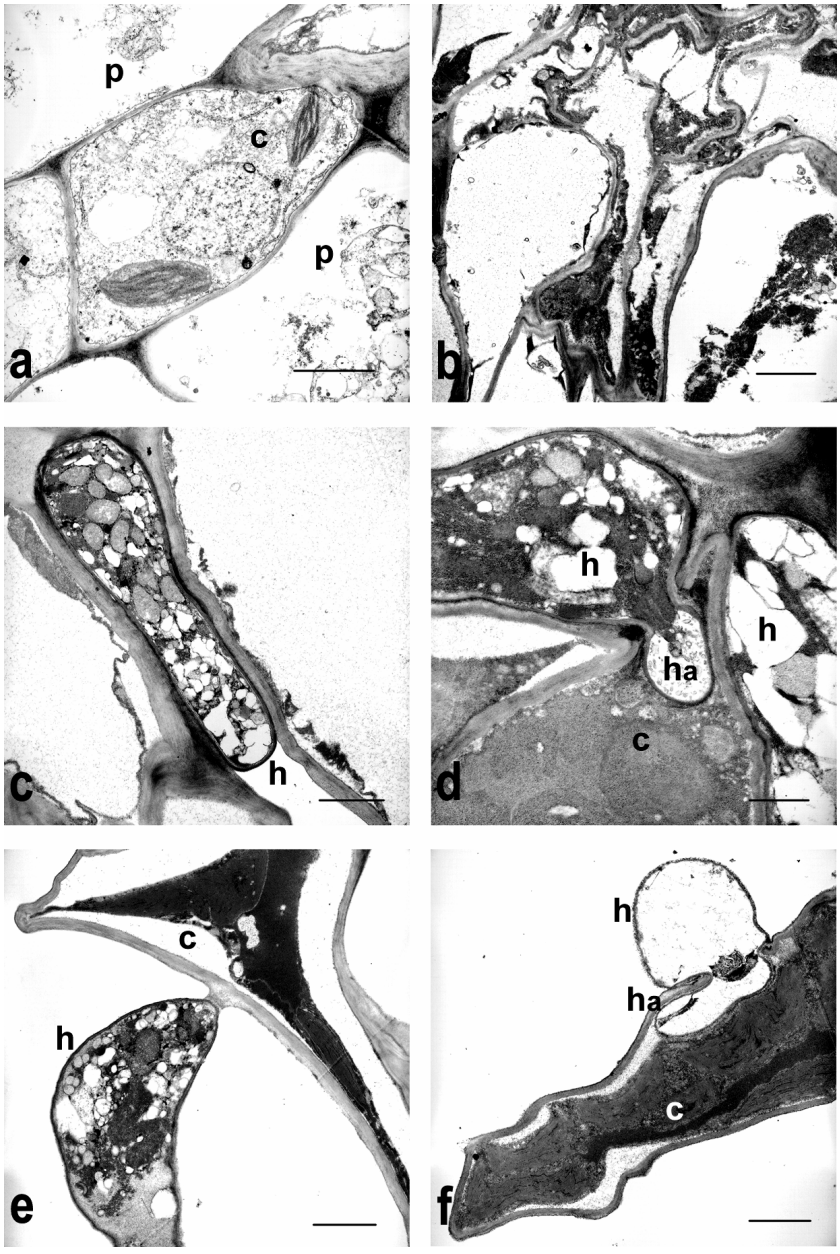

Figure 5. Transmission electron microscopy observations of young strawberry leaves artificially inoculated with *Phytophthora cactorum*. (a) Section of a non-inoculated leaf, with healthy chlorenchyma and parenchyma cells (scale bar: 2 μm). (b) Necrosed zone margin of a leaf three days after *P. cactorum* inoculation, with deteriorated parenchyma cells near the vascular tissue (scale bar: 2 μm). (c) Section of a leaf six days after inoculation of *P. cactorum*. Mature hyphae are observed in intercellular spaces in leaf tissues (scale bar: 2 μm). (d) Mature hypha forming an haustorium and penetrating into a chlorenchyma cell, in which organelles have become more electro-dense compared to organelles of healthy cells (scale bar: 1 μm). (e) Mature hypha adhered to a chlorenchyma cell, which is plasmolysed and its organelles are highly degenerated (scale bar: 2 μm). (f) Transversal section of a young hypha forming an haustorium and penetrating into a deteriorated chlorenchyma cell (scale bar: 2 μm). p, parenchyma cell; c, chlorenchyma cell; h, hypha; ha, haustorium.

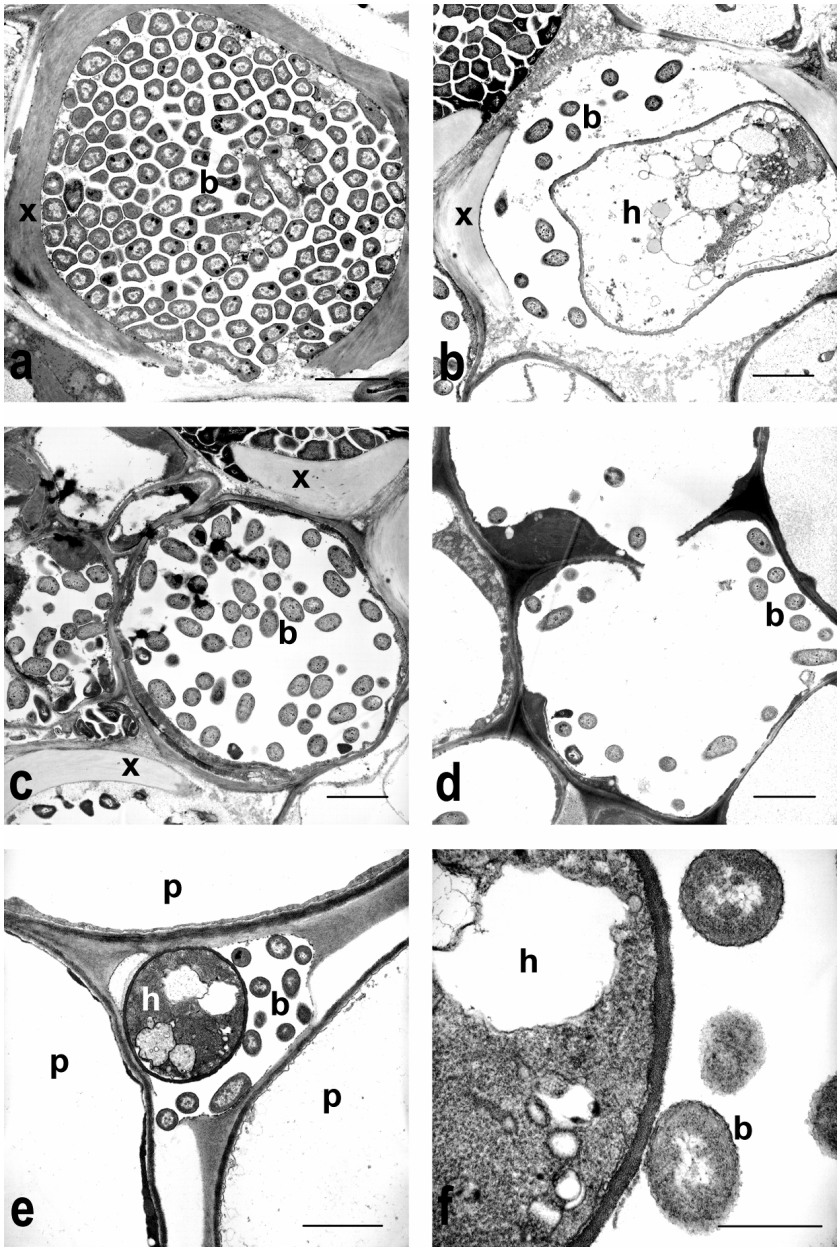

Figure 6. Transmission electron microscopy observations of young strawberry leaves treated with *Pseudomonas fluorescens* EPS894 and artificially inoculated with *Phytophthora cactorum*. (a to d) Sections of the inoculation point six days after inoculation of the pathogen showing: a xylem vessel full of bacteria (a), *P. cactorum* hypha and bacteria inside a xylem vessel (b), bacteria inside broken parenchyma cells adjacent to xylem vessels in the vascular tissue (c), and some bacteria inside broken parenchyma cells (d) (scale bars: 2 μm). (e) Intercellular space between parenchyma cells with a *P. cactorum* hypha and bacteria together (scale bar: 1 μm). (f) Detail of the close contact between bacterial cells and the hyphal surface (scale bar: 500 nm). b, bacteria; x, xylem vessel; p, parenchyma cell; h, hypha.

Discussion

P. cactorum is a soil-borne pathogen that naturally infects its host by penetrating the root, subsequently developing within the host tissues, but it can also infect leaves and fruits if they are in contact with infested soil or water (Erwin and Ribeiro, 1996). The ability of this pathogen to infect multiple host organs was the reason why detached leaves and petioles were tested as a model system to study *P. cactorum*-strawberry interactions for their use in *ex vivo* assays. *P. cactorum* was artificially inoculated by wounding the leaf or the petiole and permitting direct penetration of the pathogen into plant tissues, thus allowing an immediate host colonization and infection development. In leaves, infection necrosis progressed slower and more gradually, and differences in necrosis levels were more visible than in petioles, where necrosis progressed faster along the vascular system, collapsing it, and intermediate necrosis levels were difficult to discern, and hence results were less consistent. On the basis of these results, detached leaf tests could be an interesting option to perform *P. cactorum*-strawberry interaction studies instead of whole plant screening assays, not only because they permit a more consistent observation of intermediate levels of infection than other detached organs, but also because they can be performed rapidly and results can be measured in a shorter period of time (10 to 15 days). Inoculation of zoospore suspensions from 18-day-old cultures (10^4-10^5 zoospores per ml) on wounds permitted to achieve high infection levels in 10 days. In detached organs of strawberry cultivars Camarosa and Diamante, both leaf inoculation methods tested (puncture and cut) proved to be equally effective when young leaves were used, achieving high infection levels. Puncture wounds performed on the limbus were smaller than cut wounds performed on the midvein. Therefore, *P. cactorum* zoospores penetrated easily in cut wounds and colonization of leaf tissues occurred faster than in puncture wounds. A direct contact between the zoospore suspension drop and the wound was required, but in both cases, when wounds were fresh, *P. cactorum* could easily penetrate and infect. Ontogenic resistance to *P. cactorum* was observed in strawberry cultivars. Young leaves were more susceptible than mature and old leaves, which develop thicker cell walls by means of suberization processes and accumulate more antifungal compounds, such as phytoalexins, becoming consequently less sensitive to infection (Kus *et al.*, 2002). For this reason, young plant material is more suitable for *ex vivo* studies since host tissues are not fully developed and therefore host ontogenic resistance cannot interfere in pathogen-biocontrol agent interactions.

Light and transmission electron microscopy observations demonstrated that *P. cactorum* is able to penetrate, colonize and infect strawberry leaf tissues since a progressive tissue deterioration and cell plasmolysis was observed three and six days after pathogen inoculation. Chloroplasts and other organelles became disorganized, more electro-dense and their ultrastructure changed. These changes in cell ultrastructure can be compared with those reported by other authors in different host plant tissues infected by plant-pathogenic fungi (López-Carbonell *et al.*, 1998; Lherminier *et al.*, 2003). *P. cactorum* hyphae grew from the inoculation point through intercellular spaces and xylem vessels, and formed haustoria that penetrated the cell wall of chlorenchyma and parenchyma cells, which became more deteriorated and plasmolysed.

Differences in virulence among *P. cactorum* strains were more evident in detached leaves than in whole plant assays, and *P. cactorum* strain 489 was selected as a virulent strain to perform further studies. Additionally, differences in cultivar susceptibility were also observed in *in planta* assays. Cv. Diamante was more susceptible to *P. cactorum* infection than cv. Camarosa, as it has been reported by other authors (Browne *et al.*, 1999; Browne *et*

al., 2001; Martin and Bull, 2002). However, when detached leaves were inoculated with virulent pathogen strains, both cultivars were similarly susceptible.

The detached leaf inoculation method was effective to evaluate biocontrol activity of bacterial strains against *P. cactorum*, and permitted to find out and select strains that reduced infection severity more than 50%, in comparison with non-bacteria-treated leaves. Bacterial strains tested in potted plants behaved in a similar way as in the *ex vivo* screening. However, strain *P. fluorescens* BL915 increased disease severity in comparison with non-treated plants. This bacterial strain that was effective against *P. cactorum* on detached leaves but not in whole plants may have specific requirements that can be found in leaf tissues but not in the strawberry plant rhizosphere. It is possible that this strain does not affect the penetration process of *P. cactorum* into the plant or its development within, or it may even have a deleterious effect on the plant, which becomes more susceptible to pathogen infection. Consistency of the other bacterial strains in infection reduction in *ex vivo* and *in planta* assays may indicate that these bacterial strains operate by a biocontrol mechanism which is active on detached leaves as well as in the rhizosphere of strawberry plants. In a similar way, other authors observed that effective biocontrol agents in the field were also able to suppress the pathogen under *ex vivo* conditions. For example, biocontrol strains that suppress *Erwinia amylovora* in the field can also suppress this pathogen directly on detached flowers. But the application of high pathogen inoculum levels on detached flowers masked the efficacy of the biocontrol agents, and these biocontrol agents selected from *ex vivo* assays proved to be even more effective when tested again on a larger scale in the field with a more natural method of inoculation with the pathogen (Mercier and Lindow, 2001).

It must be pointed out that *P. fluorescens* strains EPS599 and EPS894, which highly and consistently reduced *P. cactorum* infection *ex vivo* and in whole plants, were selected and studied in more detail in the second part of Chapter I.

P. fluorescens EPS894, a strain that highly inhibited *P. cactorum* on detached strawberry leaves and whole plants, was used to observe interactions between bacterial cells and *P. cactorum* within leaf tissues. When observed with transmission electron microscopy, sections of detached leaves treated with *P. fluorescens* EPS894 and artificially inoculated with *P. cactorum* showed a successful colonization of xylem vessels by this bacterial strain. Bacterial cells were observed near and in close contact with pathogen hyphae. Cells and tissues of bacteria-treated leaves did not appear as deteriorated as those of non-bacteria-treated leaves, showing a consistency with the low infection severity levels reached in the *ex vivo* assay. This strain may reduce *P. cactorum* growth in leaf tissues via antibiosis by production of a toxic metabolite or another related mechanism. Moreover, it has been observed that, under some nutritional conditions, EPS894 is able to synthesize the antibiotics phenazine-1-carboxylic acid (PCA) and pyoluteorin (see Chapter I, second part). Therefore, the observed infection reduction in treated leaves may be caused by a metabolite produced by EPS894, probably PCA or pyoluteorin, which could interfere with the hyphal growth, although this is unclear and further studies should be performed in order to demonstrate it.

Ex vivo screening methods for chemicals or biological control agents are rapid, useful and time-saving. However, when the biocontrol mechanism consists of induction or enhancement of plant resistance against the pathogen, greenhouse or field screening methods using the whole plant are more accurate (Kloepper, 1993). In spite of this, some detached organ tests have been reported as a valid method to evaluate induction of host resistance against a pathogen prior to confirmation in greenhouse trials (Zhang *et al.*, 2002). The

proposed detached leaf inoculation method developed in this study provides consistent results when used as a screening test for biological control agents prior to greenhouse or field trials, thus allowing reduction of plant material, space and time. This method also permits the evaluation of pathogen virulence in the *P. cactorum*-strawberry pathosystem and the observation of pathogen-host interactions at a cellular level.

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Biocontrol Efficacy of *Phytophthora* Root Rot in Strawberry by *Pseudomonas fluorescens* Strains with Different Mechanisms of Action and their Combinations

Abstract

Pseudomonas fluorescens strains EPS599, EPS817 and EPS894, which were selected for their good performance on disease suppression and because they produce different antifungal metabolites, were tested individually and combined for suppression of *Phytophthora cactorum* on strawberry plants. EPS599 synthesized pyoluteorin (Plt) and showed chitinolytic activity, EPS817 synthesized 2,4-diacetylphloroglucinol (DAPG) and HCN, and EPS894 synthesized both phenazine-1-carboxylic acid (PCA) and Plt. These strains reduced 76% *P. cactorum* cyst germination on strawberry collar root extract, whereas only EPS599 and EPS894 significantly inhibited *P. cactorum* mycelial growth on PDB medium. Scanning electron microscopy revealed significant inhibition of *P. cactorum* cyst germination on strawberry root surface. Combination of bacterial strains EPS599 with EPS817, EPS599 with EPS894, EPS817 with EPS894 and the triple combination maintained but not increased the inhibitory effect on cyst germination when cells were co-incubated with *P. cactorum* and when the pathogen was incubated in mixtures of cell-free culture extracts. Combinations of strains maintained efficacy in control of *P. cactorum* in strawberry plants compared to individual strains, but reduced variability and improved consistency between experiments, especially mixtures of EPS599 with EPS817 or with EPS894.

Keywords: *Phytophthora cactorum*, *Pseudomonas fluorescens*, *Pantoea agglomerans*, biological control, *Fragaria* x *ananassa*, germination inhibition

Introduction

The Oomycete *Phytophthora cactorum* causes crown, collar and root rot and leather rot of fruit on cultivated strawberry (*Fragaria* x *ananassa* Duchesne) under favourable environmental conditions, such as high humidity and poor soil drainage (Erwin and Ribeiro, 1996). Although diseases caused by *P. cactorum* usually occur sporadically, they can potentially cause considerable damage. Fruit losses may be of 20% to 30%, and crop loss can ascend to 50% (Ellis *et al.*, 1998). Since this pathogen propagates by running water and its oospores and other resistant structures remain in soil and are difficult to eliminate, preventive control is more effective than treatments applied after the pathogen has attacked the plant and the disease has spread.

Traditionally, the control of *P. cactorum* consisted of preventive applications of chemical pesticides, but since the prohibition of some soil disinfestant products like methyl bromide it is essential to find alternative strategies. These include cultural strategies, chemical treatments with other soil disinfestants and fungicides (Ellis *et al.*, 1998; Matheron and Porchas, 2000; De Liñán, 2003; Eikemo *et al.*, 2003; Kuhajek *et al.*, 2003; De Cal *et al.*, 2004), or the application of biological control products that have been reported as an effective strategy in strawberry and other crop systems (Kloepper, 1993; Yang *et al.*, 1994; Rodríguez and Pfender, 1997; Nielsen *et al.*, 1998; Kurze *et al.*, 2001; Martin and Bull, 2002).

Biological control of *Phytophthora* spp. by different bacteria or non-pathogenic fungi has been reported in several host plants, for example in strawberry (Kurze *et al.*, 2001; Vestberg *et al.*, 2004; Porras *et al.*, 2007a; Porras *et al.*, 2007b), raspberry (Valois *et al.*, 1996), alfalfa (Silo-Suh *et al.*, 1994; Xiao *et al.*, 2002), soybean (Xiao *et al.*, 2002), tobacco (Silo-Suh *et al.*, 1994; Cartwright and Spurr, 1998), angelica trees (Okamoto *et al.*, 2000) and citrus orchards (Steddom *et al.*, 2002).

Some biological control agents have been commercialized in the USA or in other countries to be used against *Phytophthora* spp. on various non-specific crops. Examples of these products are Companion™, a mixture of *Bacillus* sp. strains, and Mycostop™, containing *Streptomyces griseoviridis* strain K61, and can be applied in greenhouse, nursery and field crops. However, the efficacy of biological control is sometimes inadequate and variability may be high. The low consistency of biological control due to its variability makes necessary the improvement of its efficacy (De Meyer *et al.*, 1998). An approach to overcome this inconsistent performance is to include a combination of biocontrol agents in a single preparation (Steddom *et al.*, 2002; Spadaro and Gullino, 2005). Different control methods that are less effective when applied individually can be combined to improve their control efficacy and, at the same time, to widen their control range and reduce the variability in their effect, presumably because the combination of biocontrol agents has a greater variety of traits responsible of biocontrol. Biocontrol agents are influenced by environmental and biotic conditions. Since various mechanisms of control can be influenced in different ways by these conditions, if several biocontrol agents with different mechanisms of action are involved in biological control, their efficacy could be increased or at least be more consistent. Some previous studies suggest that the use of more than one biocontrol agent with different mechanisms to control one or more different pathogens may be a way to reduce variability (Raupach and Kloepper, 1998; Guetsky *et al.*, 2001; Whipps, 2001; Guetsky *et al.*, 2002; Jetiyanon and Kloepper, 2002).

The mechanisms involved in biological control are often difficult to determine because they can be affected by multiple factors, especially in field conditions, and include, among others, colonization of infection sites, competition for nutrients, induced systemic resistance (ISR), and secretion of inhibitory compounds and cell wall-degrading enzymes (O'Sullivan and O'Gara, 1992; Handelsman and Stabb, 1996; Whipps, 2001; Guetsky *et al.*, 2002; Raaijmakers *et al.*, 2002).

The aim of the present study was to select and characterize biocontrol bacteria against *P. cactorum* in strawberry. Firstly, an identification of *P. fluorescens* and *P. agglomerans* strains with biocontrol activity against *P. cactorum* based on different mechanisms of action was performed. Secondly, the putative mechanisms of action of selected strains were characterized and their biocontrol activity in plants was tested. Finally, the effect of the combination of selected strains on control of the pathogen under *in vitro* and *in planta* conditions was also studied.

Materials and Methods

Pathogen and bacterial strains

Phytophthora cactorum strain 489 isolated from strawberry lesions and provided by the Plant Health Laboratory (Huelva, Spain) was used in experiments. Sporangia and zoospores were obtained from 18-day-old cultures grown on modified V8 agar (12.5 g of tomato concentrate, 2.85 g of $CaCO_3$ and 16 g of agar per liter) (Dhingra and Sinclair, 1987) at 22 ± 1ºC under a 16-h light photoperiod (Sanyo, Tokyo, Japan). Zoospores of *P. cactorum* were collected by adding sterile distilled water in culture plates (approximately 5 ml in each) and scraping the culture

surface with a Digralsky spreader. The suspension was collected with a micropipette and cooled at 5ºC for at least 3 h to induce sporangia maturation, followed by 1 h at room temperature to let sporangia release the zoospores. The desired concentration of zoospores (10^4-10^5 zoospores per ml) was adjusted by dilution from the concentrated suspension and was determined with an haemocytometer (Thoma, Brand, Germany).

A total of 46 strains of *Pseudomonas fluorescens* and *Pantoea agglomerans* were obtained from the strain collection of the Institute of Food and Agricultural Technology (Girona, Spain) and selected for their putative biocontrol activities based upon preliminary laboratory assays. Additionally, 12 strains of *P. fluorescens* from culture collections reported in the literature as biocontrol agents were included (Table 1).

Bacteria were stored at -80ºC in 20% glycerol Luria-Bertani broth (LB) (Maniatis *et al.*, 1982), and were routinely cultured on LB agar at 23ºC prior to experiments. Bacterial suspensions were prepared from 24-h LB agar cultures grown at 23ºC inoculated into 10 ml of LB broth in culture tubes and incubated on a rotatory shaker at 150 rpm at 23ºC for 24 h. Cells were then pelleted by centrifugation at 8000 x *g* for 15 min, resuspended in sterile distilled water and the final concentration was adjusted to 10^8-10^9 cfu ml^{-1}, depending on the experiment.

Plant material and maintenance

Cold-stored strawberry plants (cv. Camarosa and cv. Diamante) were used in experiments. Plants had been maintained at approximately 2ºC for at least two months when delivered from the grower. Plants used to obtain leaves, roots and for disease suppression assays were planted in 500-ml pots containing a peat:perlite (3:1) mixture and maintained in greenhouse at 25 ± 4ºC with a 16-h photoperiod for two months.

***In vitro* antagonistic activity**

Screening for *in vitro* antagonism of the 58 bacterial strains against *P. cactorum* was performed in Müeller-Hinton agar (MH) (Oxoid Ltd., Basingstoke, UK) and potato-dextrose agar (PDA) (Difco Laboratories, MD, USA), using dual cultures with agar overlays. Overlays were prepared by mixing 3.5 ml of melted 1.5% agar with 0.5 ml of a *P. cactorum* zoospore suspension adjusted to $5x10^4$ zoospores per ml. Bacterial colonies of the putative antagonists were transferred to the overlay surface with sterile toothpicks. The agar plates were incubated at 22 ± 1ºC with a 16-h photoperiod and inhibition zones were assessed after five days of incubation. 12 replicates were made for each bacterial strain. All the strains that produced inhibition zones with a 4-mm diameter or higher in at least one medium were considered as *in vitro* antagonists.

Detached leaf assay

A detached leaf assay was performed to determine *ex vivo* inhibition of *P. cactorum* infection by 58 antagonistic bacterial strains. Young leaves of strawberry plants (cv. Camarosa and cv. Diamante) were excised from the petiole and leaflets were separated, surface disinfected by immersion in a diluted solution of sodium hypochlorite (2% active chlorine) for 1 min, rinsed three times with sterile distilled water, and placed under an air stream to remove excess water. Two wounds were made with a sterile needle on the reverse of the limbus of each leaflet. Wounded leaflets were treated by immersion in a 10^8 cfu ml^{-1} suspension of the bacterial strain and placed under an air stream. A 20-µl drop of a pathogen suspension ($5x10^4$ zoospores per ml) was inoculated onto each wound performed on leaflets. Controls consisted of non-treated leaflets inoculated with *P. cactorum*, and bacteria-treated leaflets inoculated with 20 µl of sterile distilled water. Inoculated leaflets were placed with the abaxial face up on wet sterile filter paper in

Table 1. *Pseudomonas fluorescens* and *Pantoea agglomerans* strains used in the *in vitro* antagonistic activity and biocontrol on detached strawberry leaves assays against *Phytophthora cactorum*.

P.fluorescens strain	Origin	Source	*P. fluorescens* strain	Origin	Source
EPS227	Pear root	INTEA[s]	EPS808	Rootstock root[1]	INTEA
EPS231	Pear root	INTEA	EPS809	Rootstock root[1]	INTEA
EPS263	Beech root	INTEA	EPS812	Plum root	INTEA
EPS282	Alfalfa root	INTEA	EPS817	Cherry root	INTEA
EPS290	Maize root	INTEA	EPS818	Rootstock root[2]	INTEA
EPS291	Maize root	INTEA	EPS894	Apple leaf	INTEA
EPS302	Grass root	INTEA	EPS895	Apple leaf	INTEA
EPS317	Tobacco root	INTEA	EPS944	Loquat leaf	INTEA
EPS326	Lettuce root	INTEA	CHA0	Tobacco root	G. Défago[t]
EPS328	Lettuce root	INTEA	JBR1-70	Wheat root	J. Raaijmakers[u]
EPS340	Plum blossom	INTEA	WB52	Tomato root	W. Fakhouri[v]
EPS341	Plum trunk	INTEA	Ps15	Sugarcane root	M.N. Nielsen[w]
EPS353	Pear blossom	INTEA	Ps31	Sugarcane root	M.N. Nielsen[w]
EPS384	Grass leaf	INTEA	JMP1284	Wheat root	J. Raaijmakers[u]
EPS424	Blackthorn flower	INTEA	M480R	Sugarbeet root	M. J. Bailey[x]
EPS532	Peach flower	INTEA	SBW25	Wheat root	L. Thomashow[y]
EPS537	Pear blossom	INTEA	BL915	Cotton root	S. Hill[z]
EPS539	Pear blossom	INTEA	Qc69-80	Wheat root	L. Thomashow[y]
EPS550	Cherry blossom	INTEA	Q2-87	Wheat root	L. Thomashow[y]
EPS599	Hawthorn flower	INTEA	Q4-87	Wheat root	L. Thomashow[y]
EPS807	Rootstock root[1]	INTEA			

P.agglomerans strain	Origin	Source	*P.agglomerans* strain	Origin	Source
EPS394	Cherry flower	INTEA	EPS514	Pear blossom	INTEA
EPS427	Apricot flower	INTEA	EPS519	Pear blossom	INTEA
EPS435	Quince blossom	INTEA	EPS547	Plum flower	INTEA
EPS453	Peach flower	INTEA	EPS560	Hawthorn leaf	INTEA
EPS454	Peach flower	INTEA	EPS595	Pear leaf	INTEA
EPS458	Apple blossom	INTEA	EPS607	Pear leaf	INTEA
EPS475	Peach blossom	INTEA	EPS615	Hawthorn flower	INTEA
EPS495	Peach flower	INTEA	EPS622	Apple leaf	INTEA
EPS512	Apple blossom	INTEA			

[1] *Prunus persica* x *Prunus dulcis* hybrid.
[2] *Prunus persica* x *Prunus belsiana* x *Prunus domestica* hybrid.
[s] INTEA, Institute of Food and Agricultural Technology, University of Girona, Spain.
[t] Gènevieve Défago, Institute of Plant Sciences/Phytopathology, Swiss Federal Institute of Technology, Zurich, Switzerland.
[u] Jos J. Raaijmakers, Laboratory of Phytopathology, Wageningen Agricultural University, The Netherlands.
[v] Walid Fakhouri, Universität Hohenheim, Institut für Phytomedizin, Stutgart, Germany.
[w] Mette Neiendam Nielsen, Department of Ecology and Molecular Biology, Section of Genetics and Microbiology, Royal Veterinary and Agricultural University, Denmark.
[x] Mark J. Bailey, Molecular Microbial Ecology, Institute of Virology and Environmental Microbliology, Oxford, England.
[y] Linda Thomashow, US Department of Agricultural Research Service, Root Disease and Biological Control Research Unit, Washington State University, USA.
[z] Steve Hill, Syngenta Biotechnology Inc., Research Triangle Park, NC, USA.

transparent plastic boxes and incubated at 22 ± 1ºC and a 16-h light photoperiod in a controlled environment chamber (model PGR-15, Conviron Winnipeg, MB, Canada). Infection levels were assessed five, 10 and 15 days after inoculation according to the following scale: 0= no infection, 1= up to 1/3 of the leaf infected, 2= up to 2/3 of the leaf infected, and 3= up to 3/3 of the leaf infected. Infection severity (*S*) was calculated using the following formula:

$$S = \frac{\sum_{i=1}^{n} I_i}{n \cdot 3} \cdot 100$$

where *S* is the infection severity per repetition, I_i is the corresponding severity index per inoculation, and *n* is the number of inoculated leaflets per set.

Two independent experiments were performed. Experiment 1 was performed on cv. Diamante leaves and experiment 2 on cv. Camarosa leaves. In both experiments, three sets of six leaflets per treatment were arranged in a randomized experimental design.

Strain characterization

Some of the most efficient bacterial strains against *P. cactorum* in the *ex vivo* assay (EPS599, EPS817, EPS894, BL915, Ps15, Q4-87 and SBW25) were characterized for production of secondary metabolites, cell wall-degrading enzymes, inorganic phosphate solubilization ability and differential genome polymorphisms.

1-indole-3-acetic acid (IAA) and related compounds were identified upon colony growth in LB agar amended with 5 mM l-tryptophan and overlaid with an 82-mm diameter nitrocellulose membrane disk (Brito Alvarez *et al.*, 1995). Agar plates were inoculated with bacterial cultures and incubated at 28ºC for three days. Then, the membranes were removed and placed overlaid on a Whatman filter paper impregnated with the Salkowski reagent. Strains producing IAA or analog substances were identified by a pink or red colour on the filter surface.

Siderophore production was determined using chrome azurol S (CAS) medium (Schwyn and Neilands, 1987). CAS agar plates were inoculated with the strains and incubated at 28ºC for five days. Strains exhibiting an orange halo were positive for siderophore production.

Hydrogen cyanide (HCN) production was assessed by the picrate method (Sneath, 1966). Tubes containing LB agar and a filter paper strip impregnated with 0.5% picric acid and 2% $NaCO_3$ introduced into the test tube screw cup were inoculated with the bacteria. Upon incubation at 22ºC for three days, production of cyanide was detected as a change of the filter paper colour from yellow to orange-brown.

Production of salicylic acid (SA) was identified in stationary-phase cultures (48 h, 28ºC) in SSM liquid medium (Leeman *et al.*, 1996). Cultures were centrifuged at 10,000 x *g* for 10 min, the supernatant was acidified with HCl to pH 2, and SA was extracted with $CHCl_3$ (1:1, v/v). Then, 5 µl of 2 M $FeCl_3$ and 4 ml of water were added to the organic phase. The absorbance of the purple iron-SA complex in the aqueous phase was measured at 527 nm.

The presence of biosynthetic genes for production of 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), pyrrolnitrin (Prn) and pyoluteorin (Plt) was determined by PCR using the primers and conditions previously described by Raaijmakers *et al.* (1997), Badosa (2001) and Mavrodi *et al.* (2001).

The production of SA, PCA, DAPG, Prn and Plt in strawberry collar root extract (SCRE) and PDB liquid media was assessed for *P. fluorescens* strains EPS599, EPS817 and EPS894 using high performance liquid chromatography (HPLC) and nuclear magnetic resonance (^{1}H-NMR). Liquid cultures of the bacterial strains were incubated 48 h on SCRE and PDB at 25ºC

and centrifuged 15 min at 8000 x *g*. To extract metabolites for HPLC analysis, a 10-ml aliquot of each supernatant was acidified to pH 3.0 with 10% trichloroacetic acid and was extracted with a solid-phase hydrophobic adsorbant column (RESPREP SPE Cartridges: Bonded reversed phase, C_{18}, 3 ml syringe volume). The column was washed with 0.5 ml H_2O milliQ and metabolites were disadsorbed with a 1-ml solution of acetonitrile and 15% H_2O milliQ. Samples of 20 µl were injected in a Waters HPLC (model 610, Waters, Mildford, Madison, WI, USA) equiped with an analytical column (Resteck, Pinnacle II C_{18}, 5 µm, 150 x 4.6 mm) packed with Tracer Hypersil ODS attached to a precolumn (10 x 0.4 mm). The column was eluted with a solvent system of acetonitrile:water acidified at pH 2.5 with trichloroacetic acid (40:60, v/v), at a flow rate of 1 ml min^{-1}. Measurements were performed with a detector (model 484, Waters, Mildford, WI, USA) at λ=270 nm. Under these conditions, SA, PCA, DAPG, Plt and Prn were detected respectively at 4.43, 8.85, 9.73, 5.98 and 30.12 min retention time. Metabolites extraction for NMR analysis was performed as follows. A 30-ml aliquot of each supernatant was acidified to pH 3.0 with 10% trichloroacetic acid and was extracted with a solid-phase hydrophobic adsorbant column (RESPREP SPE Cartridges: Bonded reversed phase, C_{18}, 3 ml syringe volume). The column was washed with 1.5 ml acetonitrile to disadsorb metabolites, the solution was reduced to dryness under vacuum and the dry residue dissolved in D_6-acetone and analysed by nuclear magnetic resonance (^{1}H-NMR) at 200 MHz.

Cellulase, mannanase, xylanase and β-1,3-D-glucanase production was tested on tryptone-soy agar (TSA) (Oxoid Ltd., Basingstoke, UK) amended with 1 mg ml^{-1} of specific chromogenic (azurine-dyed, cross-linked; AZCL) substrate (Megazyme, Sydney, Australia) for each enzyme test (Nielsen *et al.*, 1998). Colonies of the bacterial strains were transferred to the agar surface with sterile toothpicks. Agar plates were incubated at 23ºC for 10 days. Three replicates were made for each bacterial strain. Strains that produced homogenic blue zones were considered as positive for polymer hidrolysis activity.

Chitinolytic activity of bacterial strains was assessed using a chitin medium described by Frändberg and Schnürer (1997): 2.7 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.7 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 0.5 g of KCl, 0.13 g of $CaCl_2 \cdot H_2O$, 3 g of yeast extract, 1.5 g of colloidal chitin (Rodríguez-Kábana *et al.*, 1983) and 20 g of agar per liter. Colonies of the bacterial strains were transferred to the surface of the agar plates with sterile toothpicks. Three replicates were made for each bacterial strain. Chitinolytic bacteria were identified as colonies surrounded by clearing zones after seven days of incubation at 23ºC.

Inorganic phosphate solubilization activity was assessed using three different media described by Nautiyal (1999), which contained $Ca_3(PO_4)_2$: Pikovskaya medium, NBRIY medium and NBRIP medium (Annex I). Colonies of the bacterial strains were transferred to the surface of the agar plates with sterile toothpicks. Three replicates were made for each bacterial strain. Strains that produced transparent zones after seven days of incubation at 23ºC were considered as positive for inorganic phosphate solubilization activity.

A RAPD-PCR analysis was performed to detect natural polymorphisms in the genome of bacterial strains. Bacterial strains were grown in 1 ml of LB broth for 24 h at 25ºC. Then, cultures were centrifuged 2 min at 10000 x *g*. Pellets were resuspended in 1 ml of sterile H_2O milliQ and centrifuged 2 min at 10000 x *g*. This was repeated twice. Pellets were resuspended in 150 µl of sterile H_2O milliQ, heat lysed (10 min at 99ºC) and centrifuged 1 min at 10000 x *g*. The extracted DNA was dilluted 1/100 and stored at -20ºC for later use. Amplification reactions were performed in a final volume of 25 µl

containing 1X PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.4 µM of primer, 1.5 U of *Taq* DNA polymerase (Invitrogen, CA, USA) and 5 µl of the extracted DNA. The primer used was Phl2b (5'ACCGCAG CATCGTGTATGAG3') (Raaijmakers *et al.*, 1997). RAPD fingerprints were obtained using the thermal cycle conditions described by Keel *et al.* (1996) with a minor modification: 2 cycles of 94ºC for 30 s, 36ºC for 30 s, and 72ºC for 120 s; 20 cycles of 94ºC for 20 s, 36ºC for 15 s, 45ºC for 15 s, and 72ºC for 90 s; 19 cycles of 94ºC for 30 s (incubation time increased 1 s per cycle), 36ºC for 15 s, 45ºC for 15 s, and 72ºC for 120 s (incubation time increased 3 s per cycle); and a final incubation at 72ºC for 10 min. The amplified products were visualized by gel electrophoresis in a 2% agarose gel with 1X TAE and stained with ethidium bromide.

Interaction studies between *P. cactorum* and bacterial antagonists

1. Germination inhibition

The effect of *P. fluorescens* EPS599, EPS817, EPS894 and their combinations on cyst germination was determined on strawberry collar root extract (SCRE). The effect of nutrient depletion and/or metabolites production by the antagonists on germination and growth of the pathogen was determined as described by Janisiewicz *et al.* (2000). To prepare the SCRE, a collar from a healthy young strawberry plant was grinded in a mortar and suspended in 50 ml of distilled water. After discarding the plant fragments, the suspension was sterilized by filtration with a 0.22-µm pore size membrane filter and kept at 4ºC in darkness. 24-well tissue culture plates with cylinder inserts provided with a filter membrane of 0.45-µm pore size (Millicell-CM, Millipore, Bedford, MA, USA) inside each well were used. In each well and outside the insert 0.5 ml of SCRE and 0.1 ml of sterile distilled water or bacterial suspension were added, depending on the treatment, whereas inside the insert 0.3 ml of an encysted zoospore suspension of *P. cactorum* adjusted to 10^4 zoospores per ml and 0.1 ml of sterile distilled water or bacterial suspension were added, depending on the treatment. Zoospores were encysted by agitating the zoospore suspension in a vortex shaker for 40 s. Bacterial suspensions were prepared using 48-h bacterial cultures on SCRE, which were centrifuged 20 min at 4000 *g*, pellets were resuspended in sterile distilled water and adjusted to 10^8 cfu ml^{-1}.

Five different treatments were tested (Table 2). The first treatment consisted of the co-incubation of the *P.cactorum* suspension and an individual or combined bacterial strain suspension inside the insert (in the same compartment). The second treatment consisted of the incubation of the *P. cactorum* suspension inside the insert and an individual or combined bacterial strain suspension outside the insert (in two different compartments separated by the insert membrane). The third treatment consisted of the incubation of the *P. cactorum* suspension inside the insert and a cell-free bacterial culture extract outside the insert. When single strains were tested, this cell-free culture extract corresponded to the extract of a single bacterial strain culture, whereas when combinations of strains were tested, a mixture of two or three cell-free culture extracts from two or three single bacterial strain cultures was added. The fourth treatment consisted of the incubation of the *P. cactorum* suspension inside the insert, and a cell-free bacterial culture extract from a single bacterial culture amended with glucose minimum medium (GMM) outside the insert. To prepare cell-free culture extracts, 48-h bacterial cultures on SCRE were centrifuged 20 min at 4000 x *g*. Supernatants were separated from pellets and filtered (0.22-µm pore size). GMM contained per liter: 5 g of glucose, 1 g of NH_4Cl, 3 g of KH_2PO_4, 2.4 g of Na_2HPO_4, 0.5 g of NaCl, and 0.2 g of $MgSO_4 \cdot 7H_2O$, adjusted at pH 6.5. Finally, a non-bacteria-treated control was performed with *P. cactorum* on SCRE. Culture plates with the cylinder inserts placed in each well were

sealed with parafilm and incubated at 20ºC with a 16-h photoperiod. Observations of the insert membranes were performed 24 h after addition of *P.cactorum*. Germination was stopped by adding a 20-µl drop of lactophenol blue in each cylinder insert. Then, the cylinder inserts were removed from wells and their membrane was blotted by the bottom side with tissue paper until all the liquid from the inside of the cylinder was absorbed. Thereafter, cysts were stained with lactophenol blue and membranes were cut with a sharp scalpel, transferred onto a glass slide and observed under a light microscope at 200X to determine cyst germination. Cysts appeared round and measured approximately 5 µm diameter, and germinated cysts were considered when germ tubes were longer than the diameter of the cyst. The mean percentage of germinated cysts at the beginning of the experiment was substracted from the total germinated cysts observed at the end of the experiment. Each treatment was replicated three times and the experiment was repeated twice.

2. Mycelial growth inhibition

The effect of *P. fluorescens* EPS599, EPS817 and EPS894 on *P. cactorum* mycelial growth was determined on potato-dextrose broth (PDB). Four different treatments were tested. The first treatment consisted of erlenmeyer flasks (100 ml) containing 10 ml of PDB, 4 ml of sterile distilled water, 1 ml of a 10^9 cfu ml^{-1} bacterial suspension and 5 ml of a *P. cactorum* zoospore suspension at $5x10^4$ zoospores per ml. The second treatment consisted of erlenmeyer flasks containing 10 ml of a cell-free bacterial culture extract, which was the filtered

Table 2. Treatments used to study the effect of cells and cell-free culture extracts of *P. fluorescens* strains EPS599, EPS817, EPS894 and their combinations on *P. cactorum* cysts germination when incubated in direct contact or separated by a permeable membrane in an *in vitro* well-insert complex.

Treatment	Insert	Cyst suspension (0.3 ml)	Bacterial suspension (0.1 ml)	Distilled water (0.1 ml)	SCRE medium (0.5 ml)	Cell-free extract (0.5 ml)	GMM (0.1 ml)
Non-treated	Inside	**X**		**X**			
	Outside			**X**	**X**		
Co-incubation (direct contact)	Inside	**X**	**X**				
	Outside			**X**	**X**		
Separated incubation	Inside	**X**		**X**			
	Outside		**X**		**X**		
Cell-free culture extracts	Inside	**X**		**X**			
	Outside			**X**		**X**	
Supplemented cell-free culture extracts	Inside	**X**		**X**			
	Outside					**X**	**X**

SCRE, strawberry collar root extract; GMM, glucose minimum medium.

supernatant of a centrifuged 48-h bacterial culture on PDB, 5 ml of sterile distilled water, and 5 ml of a *P. cactorum* suspension at $5x10^4$ zoospores per ml. The third treatment consisted of erlenmeyer flasks containing 10 ml of a cell-free bacterial culture extract amended with 1 ml of glucose minimum medium (GMM), 4 ml of sterile distilled water, and 5 ml of a *P. cactorum* suspension at $5x10^4$ zoospores per ml. The fourth treatment consisted of erlenmeyer flasks containing 5 ml of a pathogen suspension at $5x10^4$ zoospores per ml, 10 ml of PDB and 5 ml of sterile distilled water. Each treatment was replicated three times and the experiment was repeated twice. Dry weight of *P. cactorum* mycelia was determined seven days after incubation on a rotatory shaker at 25ºC following filtration through Whatman no. 1 filter paper and drying overnight at 80ºC.

Biological control of *P. cactorum* on strawberry plants

Two independent experiments were conducted on cv. Diamante strawberry plants to test *P. fluorescens* strains EPS599, EPS817, EPS894, BL915, Ps15, Q4-87 and SBW25. In both experiments, a total of five bacteria applications were performed. First treatment was performed at the moment of planting by dipping each plant root system in a bacterial suspension adjusted to 10^9 cfu ml^{-1}. Treated plants were introduced into 500-ml pots containing a peat:perlite (3:1) mixture and maintained in greenhouse at 25 ± 5ºC and a 16-h photoperiod during the experiments. Four additional bacteria treatments were performed on the strawberry plants seven, 21, 32 and 56 days after first treatment by irrigation with 8 ml of 10^9 cfu ml^{-1} suspensions. Non-treated control plants were irrigated with distilled water.

P. cactorum was inoculated on potted plants 28 days after first treatment. In the first experiment, plants were inoculated by irrigation with 20 ml of a *P. cactorum* suspension adjusted to $2x10^5$ zoospores per ml. In the second experiment, plants were inoculated with the pathogen by placing 40 ml of an infested substrate (approximately $1x10^6$ propagules per plant) around the roots. The *P. cactorum*-infested substrate was prepared by placing mycelium plugs of a 15-day-old *P. cactorum* culture grown on modified V8 agar in a sterilized 10-liter mixture of wheat bran:vermiculite (1:1) containing 600 g of soybean flour and 1700 ml of distilled water. The inoculated substrate was incubated at 22 ± 1ºC and a 16-h light photoperiod for 28 days, as described by Kurze *et al.* (2001), reaching approximately $5x10^4$ propagules per ml of inoculum. After *P. cactorum* inoculation, potted plants were kept in transparent plastic bags for 24 h to reach high relative humidity and favour infection by the pathogen. Holes were made in the plastic bags to avoid excessive CO_2 accumulation. Plants were maintained in greenhouse at 25 ± 5ºC with a 16-h photoperiod.

Disease levels were assessed 45 days after inoculation by observation of the level of necrosis inside the collar root and according to the following scale: 0= no necrosis, 1= up to 1/3 of the collar necrosed, 2= up to 2/3 of the collar necrosed, and 3= up to 3/3 of the collar necrosed. Disease severity was calculated using the formula described above for the detached leaf assay, where S is the disease severity per repetition, I_i is the necrosis index per plant, and n is the number of plants per set. In first experiment, two sets of five plants per treatment were arranged in a randomized experimental design, whereas in second experiment, three sets of five plants per treatment were used.

Additionally, the effect of individual and combined applications of strains EPS599, EPS817 and EPS894 on biological control of *P. cactorum* was assessed on strawberry plants of cultivar Diamante. Two independent experiments were conducted and in both experiments a total of five bacteria applications were performed as described above. First bacterial treatment was

performed at the moment of planting and four additional treatments were performed 15, 23, 38 and 53 days after first treatment. Non-treated control plants were irrigated with distilled water. 30 days after first treatment, each plant was inoculated with *P. cactorum* by placing 40 ml of an infested substrate around the roots (approximately $1x10^6$ propagules per plant). At the end of the assay (45 days after pathogen inoculation) necrosis levels of crown and collar root were assessed as described above. Three sets of five plants per treatment were arranged in a randomized experimental design and the experiment was repeated twice.

Fruit productivity and plant growth promotion

To assess fruit productivity and plant growth promotion of 'Diamante' strawberry plants by treatment with *P. fluorescens* EPS599, EPS817, EPS894, BL915, Ps15, Q4-87 and SBW25, two independent experiments were conducted. Both experiments were performed without pathogen inoculation and plants were maintained as described above. Non-treated control plants were irrigated with distilled water. Fruits were collected during and until the end of the experiment. Productivity was assessed as the total weight of fruits produced per plant. Plant growth parameters such as leaves and root fresh and dry weights as well as leaves and root water content were assessed at the end of the experiment. In first experiment, two sets of five plants per treatment were arranged in a randomized experimental design, whereas in second experiment, three sets of five plants per treatment were used.

Scanning electron microscopy of the interaction *P. cactorum*-bacterial cells on SCRE and root cuts

Two co-inoculation methods were performed in order to observe the interaction between *P. fluorescens* strains EPS599, EPS817 and EPS894 and *P. cactorum in vitro* and on strawberry roots.

To observe the interaction between bacterial strains and *P. cactorum in vitro*, a co-culture was performed, which consisted of co-cultures of bacterial cells of each strain and *P. cactorum* propagules in liquid medium (SCRE). In tubes containing 1.8 ml of SCRE, 0.9 ml of a *P. cactorum* suspension (10^5 zoospores per ml) and 0.3 ml of a bacterial strain suspension (10^8 cfu ml^{-1}) were added. Cultures were incubated 24 h at 20ºC with a 16-h photoperiod. Preparation of co-culture suspensions for scanning electron microscopy (SEM) was performed as following. In each step, suspensions were centrifuged at 7000 x *g* for 10 min and pellets were subjected to fixation and dehydratation. For standard fixation, pellets were fixed for 3 h at room temperature in glutaraldehyde (2.5%, v/v, in 0.1 M cacodylate buffer, pH 7.2) and rinsed two times in 0.1 M cacodylate buffer and finally in demineralized water. Samples were dehydrated by a series of ethanol rinses (50% to 100%), subjected to critical point drying and mounted on metal stubs (12 mm diameter) with double-sided adhesive tape.

To observe the interaction between bacterial strains and *P. cactorum* on the strawberry root surface, the following procedure was performed. A healthy strawberry plant (cv. Diamante) was removed from its pot, and roots were washed with tap water and surface disinfested by immersion in a diluted solution of sodium hypochlorite (2% active chlorine) for 1 min, rinsed three times with sterile distilled water, and placed on sterile filter paper under an air stream to remove excess water. Roots were split and immersed in tubes containing a 10^8 cfu ml^{-1} suspension of each bacterial strain for 1 h. The non-treated control consisted of roots immersed in sterile distilled water. Then, roots were immersed in a *P. cactorum* suspension adjusted to 10^5 zoospores per ml and incubated 24 h at 20ºC with a 16-h photoperiod in a controlled environment

chamber. After this, roots were prepared for SEM. Roots were gently removed from tubes and sectioned 1 cm above the root tip. Root sections were fixed and dehydrated as described for culture samples, but without the centrifugation steps. Afterwards, root samples were subjected to critical point drying and mounted on metal stubs with double-sided adhesive tape.

After being coated with a thin gold layer with a SEM coating unit (Emitech K550 Sputter Coater, Quorum Technologies, UK), culture and root samples were directly examined with a scanning electron microscope (Zeiss DSM960A, Carl Zeiss Inc., Germany) operating at 15-20 kV.

Data analysis

Pearson's coefficient of correlation was calculated for *in vitro* antagonism and *ex vivo P. cactorum* inhibition data. All data were analysed by analysis of variance (ANOVA) and the treatment means were separated by using Fisher's protected least significant difference (LSD) test or Duncan's multiple range test at $P \leq 0.05$. The coefficient of variation between experiments was calculated for all combined and single treatments in the biocontrol assay. The analysis was performed with the GLM procedure of the SAS software (version 8.2, SAS Institute, Cary, NC, USA).

Results

In vitro antagonism against *P. cactorum* and infection inhibition on detached strawberry leaves

53% of 58 bacterial strains tested had antagonistic activity against *P. cactorum in vitro* on both PDA and MH media. Bacterial strains that reduced infection severity on detached strawberry leaves more than 40% were considered as positive for *ex vivo* antagonistic activity. 69% of bacterial strains inhibited *P. cactorum* infection on detached strawberry leaves of cv. Camarosa whereas 55% of bacterial strains inhibited *P. cactorum* infection on detached leaves of cv. Diamante, reaching an infection severity lower than 40%. Leaves treated with *P. fluorescens* EPS817 and EPS894 reached very low infection levels, near 0%. Pearson's coefficient of correlation between infection severity on detached leaves treated with bacterial strains and *in vitro* antagonism against *P. cactorum* was very low for both cultivars, being r=0.0996 (cv. Diamante) and r=-0.1353 (cv. Camarosa) (Figure 1).

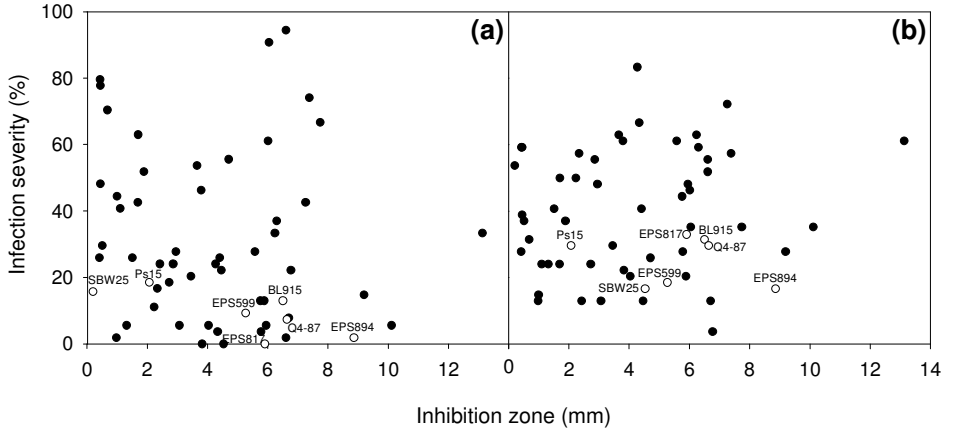

Figure 1. Relationship between infection severity on bacteria-treated (a) 'Camarosa' and (b) 'Diamante' detached leaves inoculated with *P. cactorum*, and inhibition of *P. cactorum* growth *in vitro* by 58 bacterial strains (black dots). White dots represent selected strains.

From these results, a set of seven bacterial strains that showed high efficacy in the reduction of infection severity on detached leaves but with different *in vitro* antagonistic activity were selected for further studies in order to find effective biocontrol agents with different mechanisms of action to be combined.

Characterization of selected bacterial strains

Selected *P. fluorescens* strains EPS599, EPS817, EPS894, BL915, Ps15, Q4-87 and SBW25 were characterized for production of secondary metabolites and other molecules, cell wall-degrading enzymes, inorganic phosphate solubilization ability and differential genome polymorphisms (Table 3). All bacterial strains produced siderophores but none produced the phytohormone IAA. Strains EPS817, BL915, Ps15 and Q4-87 produced HCN, and strain Ps15 produced SA. Genes related to biosynthesis of DAPG were detected in strains EPS817, Ps15 and Q4-87, and genes related to biosynthesis of Plt were detected in strains EPS599, EPS894 and BL915. Genes related to biosynthesis of PCA and Prn were detected in strains EPS894 and BL915, respectively.

HPLC results showed that EPS817 produced DAPG in both PDB and SCRE media. NMR determined that EPS599 produced Plt in both PDB and SCRE media. NMR also determined that EPS894 produced PCA and Plt in PDB with a molar relationship of 2:1, whereas in SCRE the molar relationship between PCA and Plt was approximately 1:2.

Table 3. Relevant characteristics of selected *Pseudomonas fluorescens* strains that reduced infection of *Phytophthora cactorum* on detached strawberry leaves.

	EPS599	EPS817	EPS894	BL915	Ps15	Q4-87	SBW25
Synthesized compounds[x]							
HCN	-	+	-	+	+	+	-
SA[y]	-	-	-	-	+	-	-
DAPG[y]	-	+	-	-	+	+	-
PCA[y]	-	-	+	-	-	-	-
Prn[y]	-	-	-	+	-	-	-
Plt[y]	+	-	+	-	-	-	-
Chitinolytic activity	+	-	-	+	+	-	-
Phosphate solubilization[z]	+	-	-	-	+	+	+

[x] HCN, hydrogen cyanide; SA, salicylic acid; DAPG, 2,4-diacetylphloroglucinol; PCA, phenazine-1-carboxylic acid; Prn, pyrrolnitrin; Plt, pyoluteorin.

[y] For strains EPS599, EPS817 and EPS894, symbols correspond to pooled data of PCR results (Badosa, 2001) and production of metabolites in PDB and SCRE media determined by HPLC and ^{1}H-NMR; production of SA correspond to pooled data of production in SSM liquid medium, and in PDB and SCRE media determined by HPLC and ^{1}H-NMR. For strains BL915, Ps15, Q4-87 and SBW25, symbols correspond to PCR results (Badosa, 2001), and production of SA was determined in SSM liquid medium.

[z] Results correspond to the mean of all three media tested.

Strains EPS599, Ps15 and BL915 were able to hydrolyze chitin, and strains EPS599, Ps15, Q4-87 and SBW25 were able to solubilize inorganic phosphate in almost two of the three media tested (Annex II). None of these strains showed hydrolytic activity for all the cell wall polymers tested.

Results from the RAPD-PCR showed that all strains presented different genomic polymorphism patterns (Figure 2).

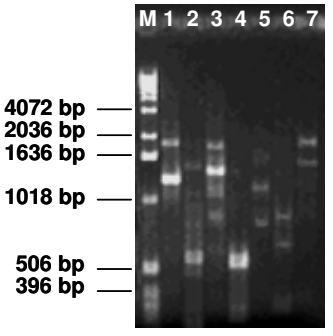

Figure 2. Genomic polymorphism patterns of selected strains amplified by RAPD-PCR with primer Phl2b. (1) EPS599, (2) EPS817, (3) EPS894, (4) BL915, (5) Ps15, (6) SBW25, (7) Q4-87, (M) 1 Kb DNA ladder marker (Invitrogen™).

Interaction between *P. cactorum* and bacterial antagonists

1. Germination inhibition

Cyst germination percentages observed on filter membranes submerged in wells containing SCRE medium after 24 h incubation was 51.0% and 59.2% in experiments 1 and 2, respectively.

Germination of *P. cactorum* cysts on SCRE was significantly reduced in both experiments when cells of *P. fluorescens* EPS599, EPS817, EPS894 and their combinations were directly interacting with cysts and germ tubes ($P<0.0001$ and $P=0.0003$, respectively), when bacterial cells were separated from cysts by a permeable membrane ($P=0.0004$ and $P=0.0012$, respectively), and when cysts were incubated with cell-free SCRE culture extracts of all three bacterial strains or with mixtures of cell-free culture extracts ($P<0.0001$).

Duncan's multiple range test was performed to assess significant mean differences among different treatments and the non-treated control for each bacterial strain and their combinations in both experiments (Figure 3). The aim of this test was to assess the putative mechanism of action of bacterial strains. In general, there were no significant differences among the effect of bacterial cells (in the same compartment or in different compartments) or cell-free culture extracts in the reduction of cyst germination produced by single bacterial strains in both experiments, except the cell-free culture extract of strain EPS894, which produced a significantly higher reduction of cyst germination compared to the effect of EPS894 bacterial cells in experiment 1. On the other hand, mixtures of cell-free culture extracts produced a significantly higher reduction of cyst germination compared to the reduction produced by co-incubation of mixtures of bacterial cells and cysts in the same compartment or in separated compartments, in both experiments. Only the combination of EPS599 with EPS817 showed a variable result, since in experiment 2 mixtures of cells of these two strains co-incubated in the same compartment showed a reduction of cyst germination significantly similar than the reduction produced by the mixture of cell-free culture extracts of these strains.

Additionally, when cell-free culture extracts of strains EPS599, EPS817 and EPS894 were amended with GMM, cyst germination increased up to approximately 50% of the total germination reached by the non-treated control. Hence, the amendment of nutrients in the cell-free culture extract, for all bacterial strains, partially restored cyst germination.

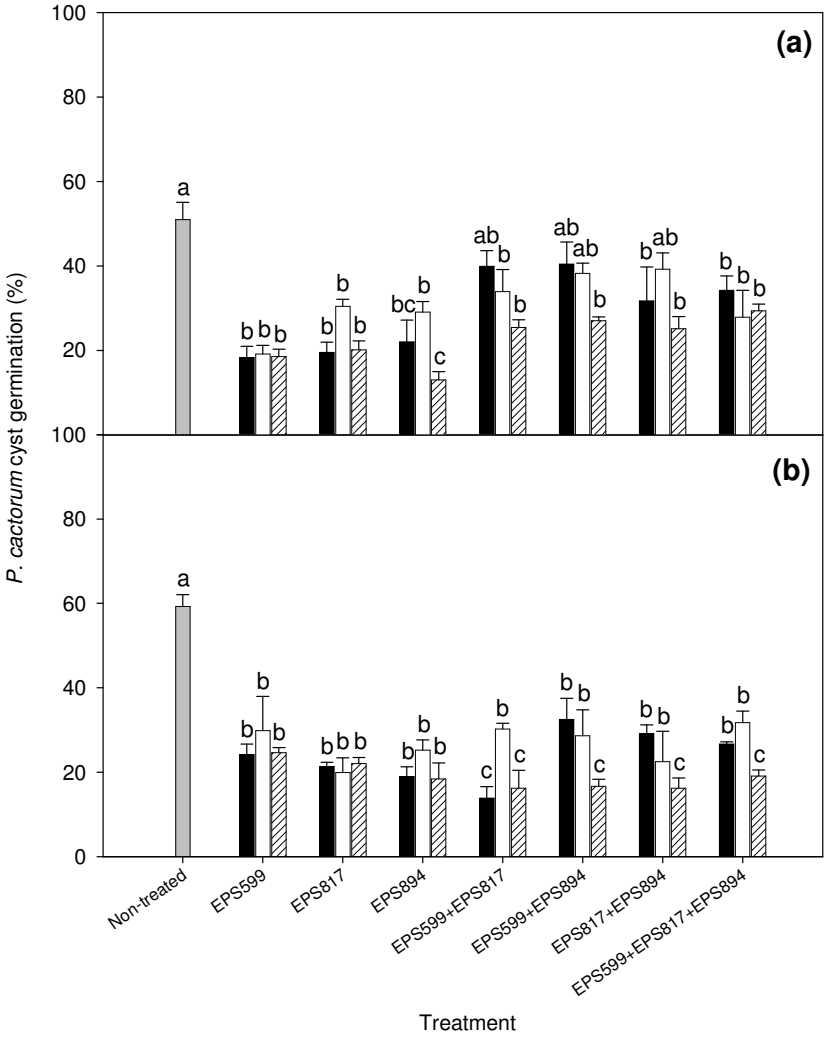

Figure 3. Effect of *P. fluorescens* strains EPS599, EPS817, EPS894 and their combinations on germination of *P. cactorum* cysts. Cysts were incubated in strawberry collar root extract (SCRE) with bacterial cells in the same compartment (black bars), separated from bacterial cells by a filter membrane (white bars) and with cell-free bacterial culture extracts (dashed bars). Non-treated controls are represented by a grey bar. Values are the mean of three replicates. Error bars represent the mean standard error. For each bacterial treatment, bars headed by different letters are significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Duncan's multiple range test was also performed to assess significant mean differences among bacterial strains and their combinations and the non-treated control for each treatment. The aim of this test was to determine if combinations of strains increased the inhibitory effect of individual strains on *P. cactorum* germination. When bacterial cells and *P. cactorum* cysts were directly interacting in the same compartment, single strains produced a significantly higher reduction of cyst germination than combinations of strains in experiment 1, but no significant differences were observed

between single strains and combinations of strains in experiment 2. Moreover, in experiment 2 the combination of strains EPS599 and EPS817 significantly reduced cyst germination compared to the rest of single and combined bacterial strains. When bacterial cells and pathogen cysts were in different compartments separated by a permeable membrane, no significant differences were observed between single strains and combination of strains in both experiments. Similarly, when pathogen cysts were incubated in cell-free culture extracts, no significant differences were observed between single strains and combination of strains in experiment 2, while in experiment 1 only the cell-free culture extract from EPS894 produced a reduction of cyst germination significantly higher than the rest of individual strains and combinations of strains.

2. Mycelial growth inhibition

Since no significant effect of experiment was observed (P=0.7783), pooled data was used. *P. cactorum* hyphal growth on PDB was significantly (P<0.0001) reduced when bacterial cells of *P. fluorescens* EPS599 and EPS894 were added (Table 4). This reduction was also observed with cell-free culture extracts. However, when cell-free culture extracts were amended with GMM hyphal growth increased, but remained significantly lower than the non-treated control. *P. fluorescens* EPS817 cells and their cell-free culture extract did not have a significant effect on reduction of *P. cactorum* mycelial weight. However, when EPS817 cell-free culture extract was amended with GMM, mycelial dry weight increased significantly, being 22% higher than the non-treated control. The mean mycelial dry weight of the non-treated controls was 67.6 mg.

Biological control of *P. cactorum* on strawberry plants

P. fluorescens EPS599, EPS817, EPS894, BL915, Ps15, Q4-87 and SBW25 were tested individually for biological control of *P. cactorum* on strawberry plants (Figure 4). Strains EPS599 and EPS894 significantly (P=0.0007) reduced disease severity levels of Phytophthora root rot in first experiment (100% and 83%, respectively) and in second experiment (100% and 87%, respectively), whereas strains BL915 and Q4-87 had no significant effect on the reduction of the disease, compared to the non-treated controls. Strains EPS817, Ps15 and SBW25

Table 4. Inhibition of mycelial growth (mg dry weight) of *P. cactorum* by *P. fluorescens* strains on potato-dextrose broth (PDB).

Treatment[1]	EPS599		EPS817		EPS894	
Non-treated	67.6	a	67.6	b	67.6	a
Co-culture	8.5	c	67.0	b	9.5	c
Cell-free culture extract	11.5	c	70.0	b	9.7	c
Cell-free culture extract+nutrients	44.7	b	93.3	a	25.5	b

[1] Non-treated: incubation of *P. cactorum* on PDB; co-culture: incubation of *P. cactorum* and bacterial strain; cell-free culture extract: incubation of *P. cactorum* on a cell-free bacterial culture extract; cell-free culture extract+nutrients: incubation of *P. cactorum* on a cell-free bacterial culture extract amended with glucose minimum medium (GMM). Mycelial growth was determined after seven days of incubation at 25ºC.
For each bacterial strain, values followed by different letters are significantly different (P≤0.05) according to Duncan's multiple range test. Values correspond to pooled data from experiment 1 and 2.

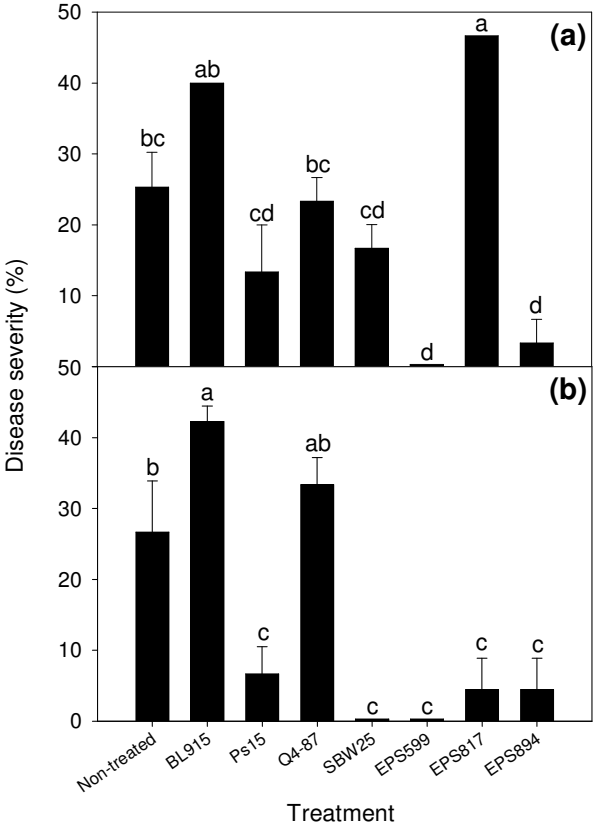

Figure 4. Effect of *P. fluorescens* strains BL915, Ps15, Q4-87, SBW25, EPS599, EPS817 and EPS894 on *P. cactorum* disease severity in potted strawberry plants of cv. Diamante. Values are the mean of two sets of five plants in experiment 1 (a) and three sets of five plants in experiment 2 (b). Error bars represent the mean standard error. Bars headed by different letters are significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

were only effective in experiment 2, with a 83%, 75% and 100% disease reduction, respectively, whereas in experiment 1 plants treated with these two strains reached disease severity levels significantly similar or higher than the non-treated control. Disease severity in non-treated control plants was low and ranged from 25% to 27% in both experiments.

Strains EPS599, EPS817, EPS894 and their combinations were tested for *P. cactorum* biocontrol efficacy on strawberry plants. All combinations of bacterial strains and also EPS599 applied individually significantly reduced disease severity, compared to the non-treated control, in both experiments, whereas EPS894 significantly reduced disease severity in only experiment 2, and EPS817 had no significant effect on reduction of disease severity (Figure 5).

Strain EPS599 produced a reduction of disease severity of 56% and 68% in experiments 1 and 2 respectively, compared to the non-treated control. A higher variability was observed in plants treated with strain EPS894, which reduced disease severity levels 67% and 42% in experiments 1 and 2 respectively, compared to non-treated controls. Application of strain EPS817 obtained a lower reduction of disease severity in both experiments (33% and 32%), compared to the non-treated control. The combination of strains EPS599 with EPS817, EPS599 with EPS894, EPS817 with EPS894 and the triple combination of

strains EPS599, EPS817 and EPS894 produced a reduction of disease severity that ranged from 56% to 78%, in both experiments.

Although combinations of bacterial strains did not improve efficacy of disease suppression compared to single strains, they reduced variability between experiments. The coefficient of variation between experiments of treatments with single strains was rather high, being 58.6%, 55.6% and 108% for strains EPS599, EPS817 and EPS894, respectively. The coefficient of variation between experiments of plants treated with mixtures of EPS599 with EPS817 and EPS599 with EPS894 was lower than in treatments with single strains (30.6% and 35.0%, respectively), whereas the coefficient of variation between experiments of plants treated with the combinations of EPS817 with EPS894 and the triple combination was higher (63.8% and 63.2%, respectively). Non-treated control plants reached disease severity levels of 40.0% and 42.2% in both experiments, and a coefficient of variation between experiments of 26.0%.

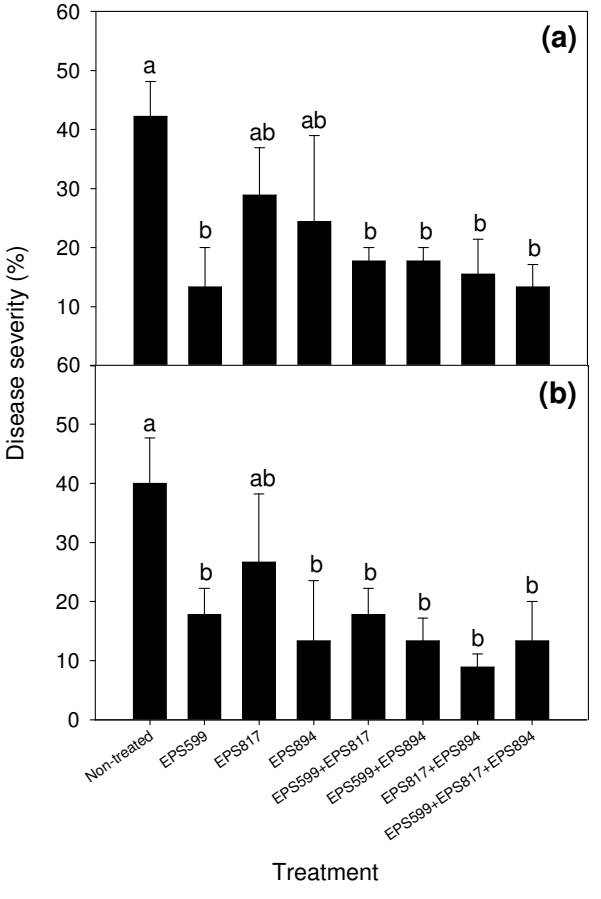

Figure 5. Effect of *P. fluorescens* strains EPS599, EPS817, EPS894 and their combinations on *P. cactorum* disease severity in potted strawberry plants of cv. Diamante. Values are the mean of three sets of five plants, and correspond to two independent experiments (a and b). Error bars represent the mean standard error. Bars headed by different letters are significantly different ($P \leq 0.05$) according to Fisher's protected least significant difference (LSD) test.

Fruit productivity and plant growth promotion

Bacterial treatments did not have a significant effect neither on strawberry fruit productivity nor on plant growth parameters, compared to non-treated controls. Nevertheless, *P. fluorescens* EPS599 and EPS894 significantly ($P<0.05$) increased root dry weight of plants compared to non-treated plants in one of the experiments (22.5% and 20.4%, respectively).

Scanning electron microscopy of the interaction *P.cactorum*-bacterial cells on SCRE and root cuts

P. fluorescens strains EPS599, EPS817 and EPS894 appeared attached to the surface of different *P. cactorum* structures when samples from *in vitro* liquid suspensions were observed with scanning electron microscopy (SEM) (Figure 6). Bacterial cells were observed on the surface of germinated cysts, especially on the cyst and on the base of the germ tube (Figure 6 a, d and e). Bacteria attached to hyphae were also observed (Figure 6 b), but quantity of bacterial cells on hyphal surface was lower than that observed on other structures. Bacterial cells were also observed partially covering the surface of oogonia and sporangia (Figure 6 c and f). Sporangia that had not released their zoospores and were completely covered by bacterial cells were frequently observed. In samples of all three strains, germination of *P. cactorum* cysts was either inhibited or reduced, and germ tubes appeared poorly developed.

Scanning electron microscopy observations of strawberry roots treated with strains EPS599, EPS817 and EPS894, and inoculated with *P. cactorum* are showed in Figure 7. Some germinated cysts could be observed in all bacteria-treated root tips, although hyphal development was less evident than that of the non-treated control. 24 h after inoculation, high pathogen growth was observed on the surface of non-bacteria-treated control roots. *P. cactorum* encysted zoospores germinated and formed dense hyphal growth around the root. Some hyphae grew into the creases and grooves of the root epidermis, presumably to penetrate and infect the root (Figure 7 a). A detailed view of cysts and hyphae on the root surface permitted the appreciation of the hyphal tips adhered to the root surface (Figure 7 f), whereas poor hyphal development was observed on all bacteria-treated roots (Figure 7 b, c and d). A magnification of a root treated with strain EPS894 showed presence of bacterial cells close to the pathogen in epidermal creases. Bacterial cells were also observed embedded in a mucilaginous matrix on the root surface, forming microcolonies (Figure 7 e and g).

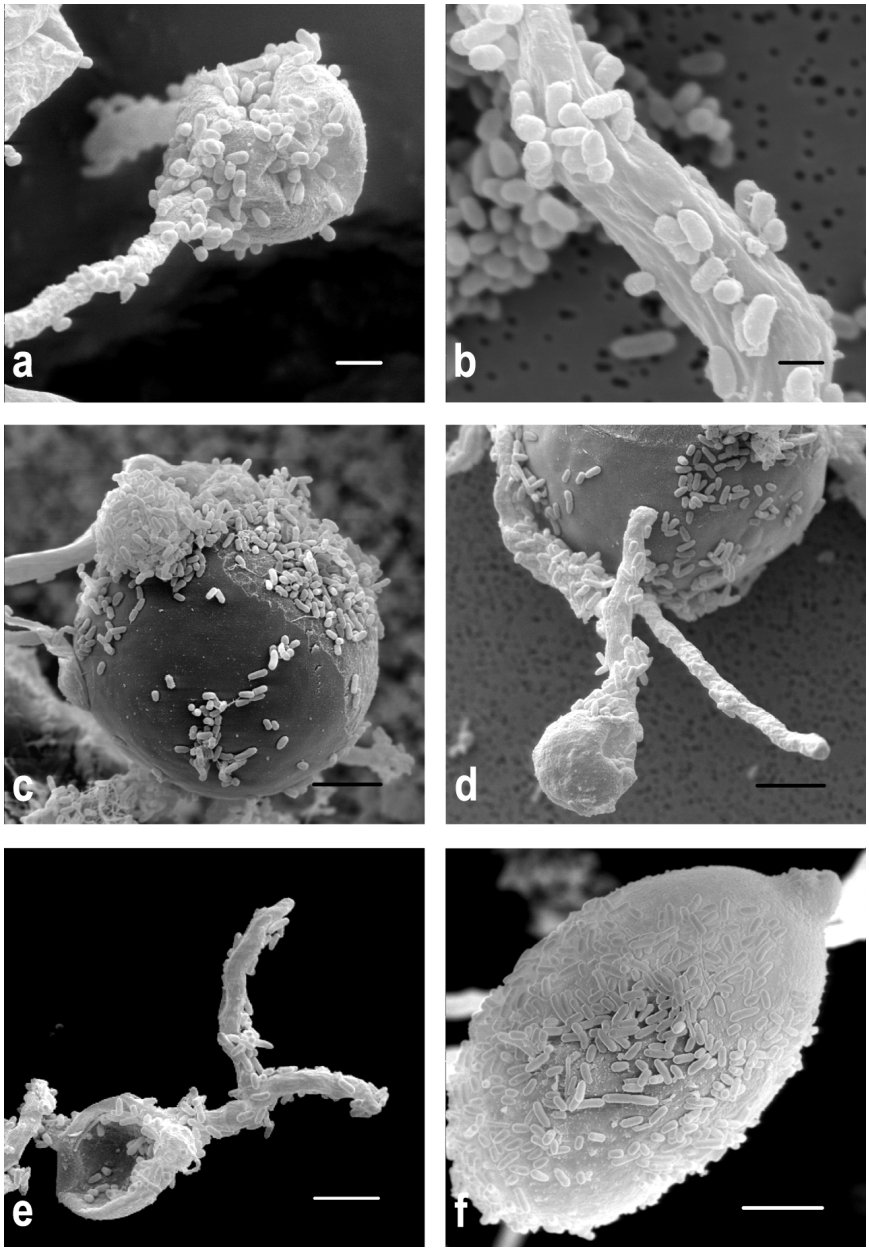

Figure 6. Scanning electron micrographs of *P. fluorescens* strains EPS599, EPS817 and EPS894 interacting with *P. cactorum* propagules induced to germinate *in vitro*. Micrographs were taken 24 h after inoculation. (a) Germinated cyst with attached EPS599 cells (scale bar: 2 μm). (b) Hypha with attached EPS599 cells (scale bar: 1 μm). (c) Oogonium and antheridium with EPS599 cells (scale bar: 5 μm). (d) Germinated cyst and oospore with EPS817 cells (scale bar: 5 μm). (e) Germinated cyst partially covered with EPS894 cells, especially on the germinating tube (scale bar: 5 μm). (f) Sporangium with papilla completely covered with EPS817 cells (scale bar: 5 μm).

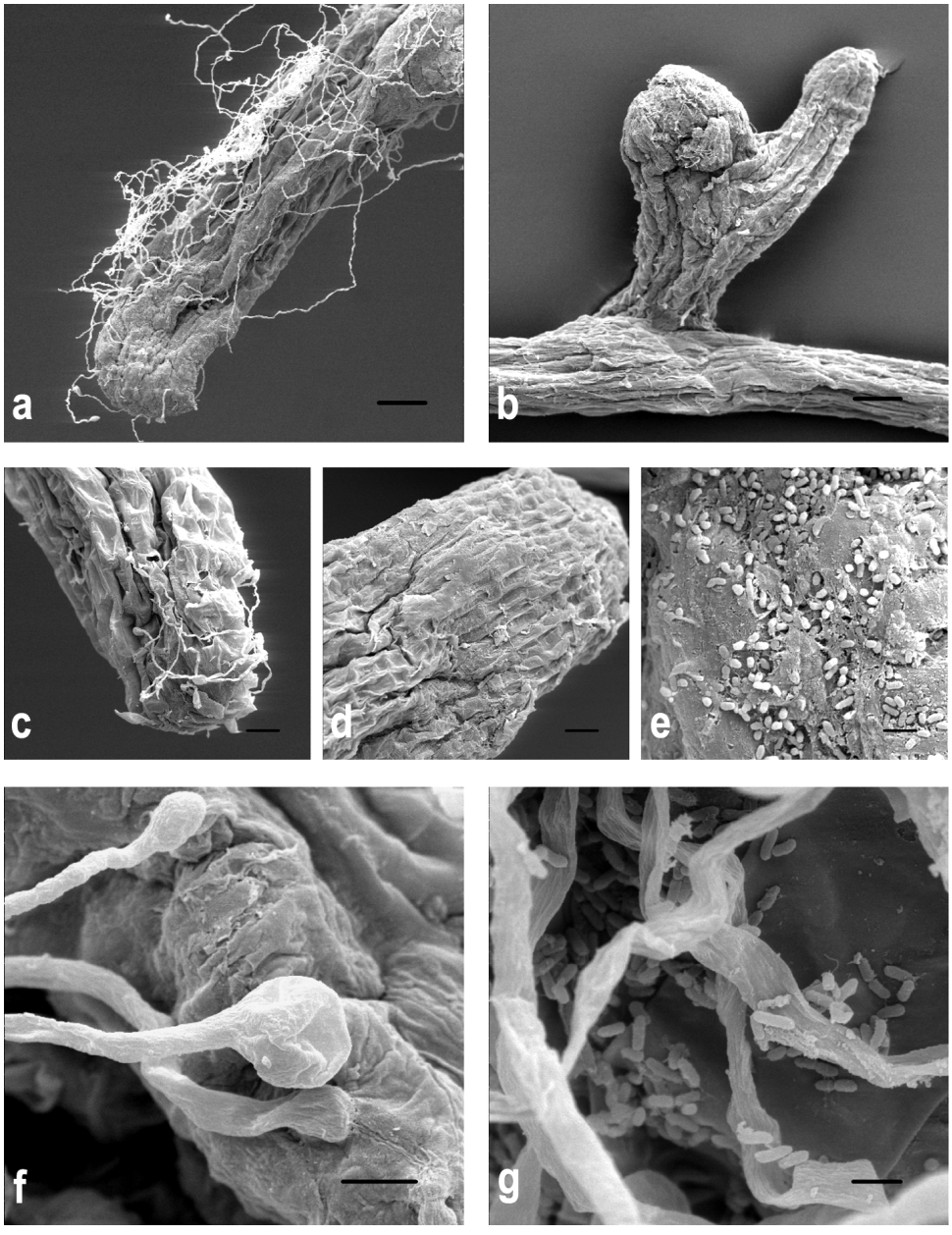

Figure 7. Scanning electron micrographs of *P. fluorescens* strains EPS599, EPS817 and EPS894 interacting with *P. cactorum* propagules germinating on strawberry roots (cv. Diamante). Micrographs correspond to 24 h after inoculation. (a) Encysted and germinated zoospores growing on the root tip surface without bacterial treatment (scale bar: 50 μm). (b) EPS894-treated root tip (scale bar: 100 μm). (c) Roots treated with EPS817 (scale bar: 20 μm). (d) Roots treated with EPS599 (scale bar: 20 μm). (e) EPS894 cells on the root surface (scale bar: 2 μm). (f) Non-treated root surface (scale bar: 2 μm). (g) Hyphae and bacterial cells on the surface of an EPS894-treated root (scale bar: 2 μm).

Discussion

Pseudomonas fluorescens EPS599, EPS817, EPS894, BL915, Ps15, Q4-87 and SBW25 clearly reduced *Phytophthora cactorum* infection severity on detached strawberry leaves of cultivars Camarosa and Diamante, and were selected because of their different levels of *in vitro* antagonism, pointing out that they may inhibit *P. cactorum* by different mechanisms, and proved to be different strains as showed by the pattern of genomic polymorphisms obtained by RAPD-PCR. The effect of bacterial strains against *P. cactorum* on strawberry was variable. The most effective strains were EPS599 and EPS894, whereas the efficacy of strains EPS817, Ps15 and SBW25 was more variable. Strains BL915 and Q4-87 did not control *P. cactorum* in strawberry plants. Three strains (EPS599, EPS817 and EPS894) were selected for their performance *in vitro*, *ex vivo* and in strawberry plants, and were characterized and furtherly studied.

P. fluorescens EPS599, which is able to synthesize Plt and siderophores and also to solubilize inorganic phosphate and hydrolyze chitin, was effective against *P. cactorum* in all assays performed *in vitro*, *ex vivo* and, especially, in strawberry plants. *P. fluorescens* EPS817 produced siderophores, HCN and DAPG in different media, and showed *in vitro* and *ex vivo* inhibition of *P. cactorum*, but its efficacy against the pathogen in strawberry plants was rather variable. *P. fluorescens* EPS894 produced PCA, Plt and siderophores, and efficiently inhibited *P. cactorum in vitro*, *ex vivo* and *in planta*. The fact that EPS894 synthesizes both PCA and Plt and EPS599 synthesizes Plt but not PCA is interesting. Liu *et al.* (2006) found that, among all the antibiotic-producing bacteria isolated from green pepper rhizosphere, mostly *Pseudomonas* spp., the population of strains that were able to synthesize both PCA and Plt was quite rare compared to the population of strains that only produced PCA, and moreover, they did not find any strain that only produced Plt. Nevertheless, the results obtained in the present study suggest that, although rare, strains that produce both PCA and Plt or Plt alone can be found in nature.

Strains EPS599, EPS817 and EPS894 significantly inhibited *P. cactorum* cyst germination when bacterial cells were co-incubated with the pathogen in strawberry collar root extract (SCRE) in the same or in separated compartments, and when the pathogen was incubated in cell-free extracts from 48-h bacterial cultures. In all treatments with individual strains, the effect of bacterial cells co-incubated with pathogen cysts in the same compartment on cyst germination was generally not significantly different from the effect produced by bacterial cells co-incubated with cysts in different compartments separated by a permeable membrane, or by the effect produced by cell-free bacterial culture extracts, suggesting a mechanism of antagonism presumably based on antibiosis. Furthermore, the inhibitory effect of cell-free culture extracts was partially restored when amended with glucose minimum medium (GMM). This putative mechanism of antagonism based on antibiosis is probably mediated by Plt in EPS599, DAPG and HCN in EPS817, and PCA and Plt in EPS894. Nutrient competition may not be the main mechanism of action of these bacterial strains but it may have some effect since addition of nutrients to cell-free culture extracts led to a partial restoration of cyst germination. This indicate that, either when bacterial cells and cysts are co-incubated in a medium with limited nutrients or when cysts are incubated in cell-free culture extracts, the inhibitory effect of bacterial cells on cyst germination is mostly due to antibiosis and, to some extent, to nutrient competition. EPS599 and EPS894 also inhibited mycelial growth when cells and cell-free culture extracts were co-incubated with *P. cactorum* in PDB. In this medium, EPS599 produced Plt and EPS894 produced PCA and Plt. However, no inhibitory effect on mycelial growth was observed in any treatment with strain EPS817 on PDB.

DAPG production was detected in both PDB and SCRE culture extracts of strain EPS817, being lower in PDB medium. Perhaps cyst germination and mycelial growth depended on DAPG concentration in the medium, and a minimum concentration threshold was necessary to obtain a significant effect on *P. cactorum* growth inhibition. Certains pathogens are inhibited when a given concentration threshold of DAPG is surpassed (Cronin *et al.*, 1997). EPS817 showed a slightly significant effect on disease severity reduction in strawberry plants, but variability between experiments was rather high. This variability may be due to variation in the production of DAPG and HCN, which would not be produced in enough quantities to inhibit *P. cactorum in situ*. This variability in effectiveness of disease reduction was also observed in the *in vitro* germination and mycelial growth inhibition assays, in which EPS817 significantly reduced cyst germination but not mycelial growth.

The effect of the co-incubation of mixtures of strains EPS599 with EPS894, EPS817 with EPS894, and the triple combination on cyst germination was not different or was slightly less effective than strains applied individually, probably because of competition between bacterial strains or by other factors, such as incompatibilities of synthesis regulation of different antibiotics between bacterial strains. Some studies of *in vitro* incompatibilities between biocontrol strains have been reported (Schnider-Keel *et al.* 2000; Lutz *et al.*, 2004). Only the combination of strains EPS599 with EPS817 increased the inhibitory effect on cyst germination *in vitro*, but only in one experiment, even though in another experiment this combination was slightly less effective than strains applied individually. Generally, mixtures of cell-free extracts from individual bacterial strain cultures had a higher effect on reduction of cyst germination compared to mixtures of bacterial cells. When cell-free culture extracts from different single strain cultures were mixed, the combination of secondary metabolites produced by these strains might increase the inhibitory effect on pathogen cysts, as there were no bacterial cells that would compete for nutrients or have other incompatibilities that would interfere with the synthesis of secondary metabolites and cyst inhibition. Scanning electron microscopy also revealed significant inhibition of *P. cactorum* cyst germination and hyphal growth by bacterial strains *in vitro* and on strawberry roots. These observations, especially of strawberry roots, confirmed the inhibitory effect of bacterial strains on cysts germination and hyphal growth that was already observed in the *in vitro* assays.

The combination of bacterial strains did not improve biocontrol efficacy of *P. cactorum* in strawberry plants, but reduced variability between experiments compared to application of individual strains. The coefficient of variation between experiments observed in plants treated with EPS894 was rather high, compared to the variation observed in treatments with EPS599 and EPS817 applied individually. Dual mixtures of EPS599 with EPS817 and EPS599 with EPS894 were the best in reducing variability. However, the combination of strains EPS817 with EPS894 and the triple combination did not produce an important reduction of variability.

Generally, application of mixtures of strains may result in higher biocontrol and lower variability of efficacy, thus increasing the reliability of biocontrol, as it has been reported in other studies on different pathosystems. Guetsky *et al.* (2001) suggested that application of more than one antagonist with different ecological requirements would increase the reliability and decrease the variability of biocontrol. In their study, combination of the yeast *Pichia guilermondii* Y2 and the bacterium *Bacillus mycoides* B16, which had different temperature and humidity optimals as well as different mechanisms of action, was effective at suppressing *Botrytis cinerea* in detached strawberry leaves under a

wider range of conditions compared to individual applications of these biocontrol agents. In another study, Guetsky *et al.* (2002) observed that *P. guilermondii* competed with *B. cinerea* for several nutrients, secreted an inhibitory compound and cell-wall degrading enzymes *in vitro* and activated the plant defenses, whereas *B. mycoides* also secreted an inhibitory compound and cell-wall degrading enzymes *in vitro* and activated systemic resistance in the plant. The combination of these two biocontrol agents on detached strawberry leaves and in whole plants increased biocontrol since the effect of their biocontrol mechanisms was cumulative. Similarly, Roberts *et al.* (2005) reported an increase of biocontrol effectiveness with certain strain combinations of *Trichoderma virens*, *Serratia marcescens* and *Burkholderia* spp. against soilborne pathogens *Rhizoctonia solani* and *Pythium ultimum* on cucumber plants.

Two or more strains that are growing together *in vitro* in a medium are probably competing for nutrients and, at the same time, synthesizing molecules that can interfere with each other's metabolism. Although combinations of bacterial strains show *in vitro* incompatibilities or do not improve germination inhibition compared to single strains, they appear to be compatible in strawberry plants and provide a higher and more consistent biocontrol effect. This may suggest that these strains are probably occupying different sites of the root in the strawberry root system and thus they do not interfere with each other. If different strains that individually have an antagonistic effect against *P. cactorum* in plants are applied combined they may improve their control efficacy and consistency by colonization of different sites of the root. Duffy *et al.* (1996) suggested that combinations of *Trichoderma koningii* with *Pseudomonas* strains that produced antifungal metabolites enhanced suppression of take-all of wheat because *T. koningii* was not inhibited by metabolites produced by *Pseudomonas* spp. since both biocontrol agents were occupying different sites in the wheat rhizosphere. Moreover, Pierson and Weller (1994) found that many of the *P. fluorescens* strains that were components of mixtures that effectively suppressed take-all disease on wheat were either strongly inhibitory to or strongly inhibited by other members of the mixture *in vitro*. These authors suggested that antagonism or incompatibility among strains may result in greater competition among these bacteria in the rhizosphere and, therefore, more consistent expression of traits involved in competition and disease control. In the present study, if bacterial strains applied as a mixture colonize different sites of the strawberry root, the probability that *P. cactorum* and one or more of the bacterial strains interact in an infection site is higher, increasing the probability of local *P. cactorum* inhibition by production of antibiotics. Combinations of strains permit a potentially more extensive colonization of the rhizosphere and provide more consistent disease suppression due to a higher probability of expression of important biocontrol traits under a broader range of environmental conditions than strains applied individually, and not only by the increased number of biocontrol agents applied (Pierson and Weller, 1994; Guetsky *et al.*, 2001; Meyer and Roberts, 2002; Roberts *et al.*, 2005).

The biocontrol mechanisms of individual bacterial strains in a mixture may be enhanced by compatible utilization of nutrients or rhizosphere colonization, and also by molecular signaling among the individual strains; therefore, it is likely that these strains act synergistically (Lutz *et al.*, 2004). So, strain combinations that do not have a lower effect on disease suppression compared to the same strains applied individually are more likely to produce a more consistent biocontrol (Roberts *et al.*, 2005).

In conclusion, *P. fluorescens* EPS599, EPS817 and EPS894 significantly reduced *P. cactorum* cyst germination *in vitro* and on strawberry roots presumably by different

mechanisms of action, being antibiosis one of these mechanisms, at least *in vitro*. Mixtures of these bacterial strains reduced variability of biocontrol in strawberry plants and seemed to improve consistency of disease suppression compared to application of single strains.

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Chapter II

Characterization and efficacy of bacterial strains for biological control of *Meloidogyne javanica* in the GF-677 rootstock

Introduction

Plant parasitic nematodes

Nematodes (Phylum Nematoda) are unsegmented round-worms and the most abundant multicellular animals on Earth. They are highly similar in structure but can be identified by their major specific morphologic variation. Nematodes occupy all ecological niches, and most species are parasites of other complex animals and plants. Only 10% of all described nematode species are plant parasites, and they infect plants growing in fields, prairies, forests, nurseries, orchards, turf, greenhouses and containers. Since the mid-1940s, plant-parasitic nematodes have been recognized as the major cause of plant biotic stress and crop loss of a wide range of economically important plant species. Most of the yield damage is caused by sedentary root-knot (e.g. *Meloidogyne* spp.), cyst (e.g. *Heterodera* spp., *Globodera* spp.), as well as several migratory endoparasitic nematodes (e.g. *Pratylenchus* spp.) (Shurtleff and Averre, 2000; Bird and Kaloshian, 2003).

The root knot nematode *Meloidogyne* spp.

The sedentary endoparasitic root-knot nematodes of the genus *Meloidogyne* are among the most successful parasites in nature (Figure 2.1), and they can infect more than 3000 plant species, including vegetables, legumes, cereals and grasses, bush and fruit trees, and herbaceous and

woody ornamentals. *Meloidogyne* spp. are distributed worldwide although the most important species, such as *M. javanica*, *M. incognita* and *M. arenaria*, are more adapted to warm and tropical climates. Except some less common species, *Meloidogyne* spp. are not very host-specific (Abad *et al.,* 2000; Pinochet and Bello, 2000; Shurtleff and Averre, 2000). Some of their biological features, such as wide host range, short generation time and reproduction via mytotic parthenogenesis, allow root-knot nematodes to reach high population levels and therefore constitute a major threat to crop production (Goverse *et al.*, 2000). The most characteristic symptom of *Meloidogyne* spp. infection is the formation of root galls or 'root knots', which alter the entire physiology of the infected plant.

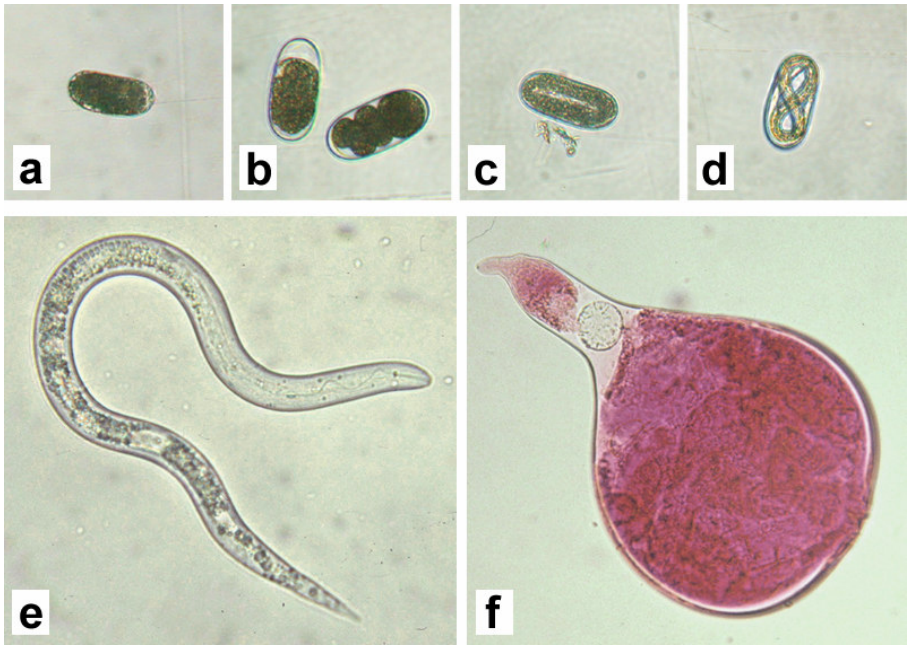

Figure 2.1. Morphology of *Meloidogyne* spp. (a to d) Nematode development inside the egg from single-cell stage to juvenile or larva stage. (e) Second-stage juvenile (J2) hatched from the egg. (f) Adult female. Images (e) and (f) are courtesy of J. Pinochet.

Life cycle of* Meloidogyne *spp.

The infection process begins when second-stage juveniles (J2) hatch from the egg, migrate through the soil to the elongation zone of the root and penetrate the root tip just behind the root cap (Figure 2.2).

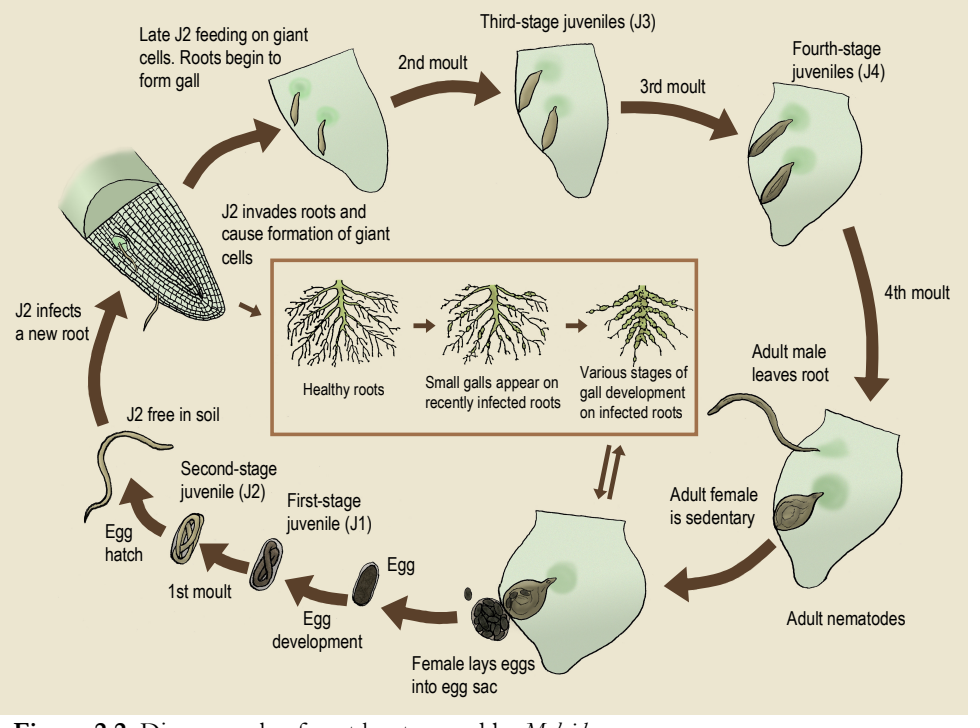

Figure 2.2. Disease cycle of root knot caused by *Meloidogyne* spp.

Juveniles enter the root via combination of mechanical penetration by the stylet and secretion of cell wall-degrading enzymes (β-1,4-endoglucanases and other enzymes) produced in subventral esophageal glands, and move intercellularly to the vascular cylinder, where they migrate up the root until they find suitable cells, usually precambial cells of the vascular parenchyma, to establish a feeding site. Upon induction by secreted signalling molecules, suitable cells develop into giant cells, which become the nematode feeding site. These nematode secretions are produced in dorsal glands and include several proteins involved in cell cycle and metabolism regulation, cytoskeleton formation and other enzymes that induce the formation of giant cells, which are the result of multiple nuclear divisions uncoupled from cytokinesis that generate metabolically active multinucleate cells formed by two to 12 parenchymatic cells.

These giant cells have multiple enlarged nuclei, small vacuoles, proliferation of smooth endoplasmic reticulum, ribosomes, mitochondria and plastids, and possess finger-like cell wall invaginations lined with plasma membrane that facilitate transport of nutrients from the vascular system into the cell. The surrounding cells of the feeding site also divide and swell to form a gall or 'root-knot' with the nematode and the giant cells embedded within. Each juvenile triggers the development of five to seven giant cells, from which the nematode obtains water and nutrients and, after three moults, develops into a pear-shaped female (Figure 2.3), or into a vermiform male under stressful environmental conditions. Approximately three weeks after development of the female, hundreds of eggs are released to the root surface in a protective, gelatinous matrix (Figure 2.3) (Williamson and Hussey, 1996; Bird and Koltai, 2000; Goverse *et al.*, 2000; Pinochet and Bello, 2000; Abad *et al.*, 2002; Gheysen and Fenoll, 2002; Bird and Kaloshian, 2003; Williamson and Gleason, 2003).

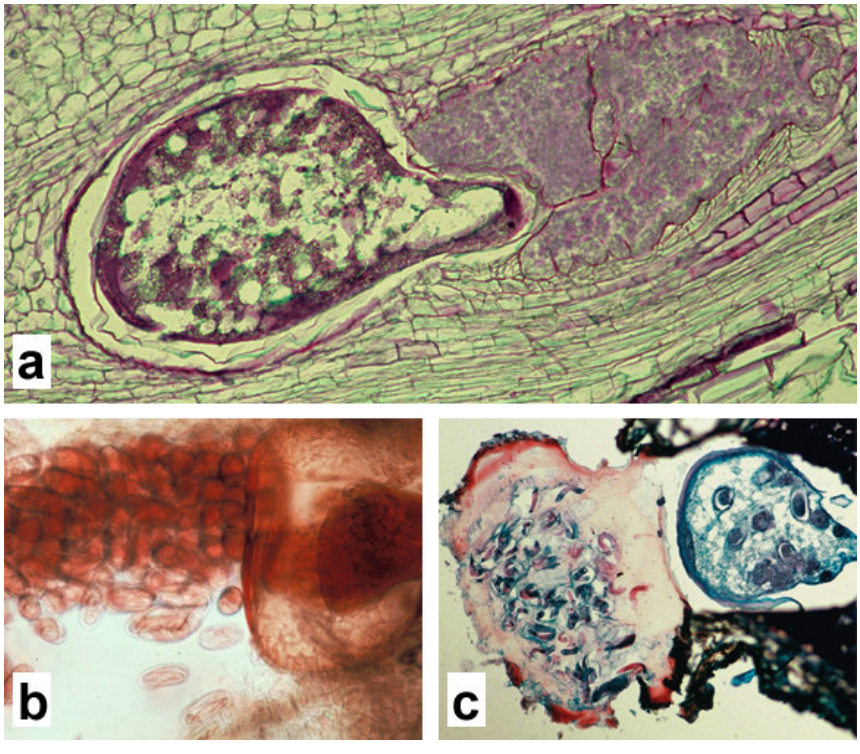

Figure 2.3. (a) Female embedded within a root gall (left) and feeding site formed by giant cells (right). (b) Female releasing eggs to the root surface in a gelatinous matrix. (c) Female (right) and eggs at different developmental stages within the gelatinous matrix (left). Images are courtesy of J. Pinochet.

Disease symptoms

Symptoms of nematode infection include poor plant development and growth, stunting, early decline, wilting, chlorosis, symptoms usually associated with nutritional and water deficiencies or excesses, susceptibility to other pathogens and, occasionally, plant death. Severely infected plants will usually wilt sooner than healthy ones when moisture is limiting. The leaves of some woody plants may be small and off-colour, especially in the upper crown. Early defoliation as well as twig and branch dieback are other common symptoms. Formation of galls causes destruction or collapse of the root vascular system of infected plants, altering the nutrient and water absortion and the entire physiology of the plant. The entire root system becomes stubby as a result of the general alteration of root growth (Figure 2.4) (Pinochet and Bello, 2000; Shurtleff and Averre, 2000).

Additionally, other pathogens, such as fungi and bacteria, may easily invade plants through injuries made by nematodes (Pinochet and Bello, 2000; Shurtleff and Averre, 2000).

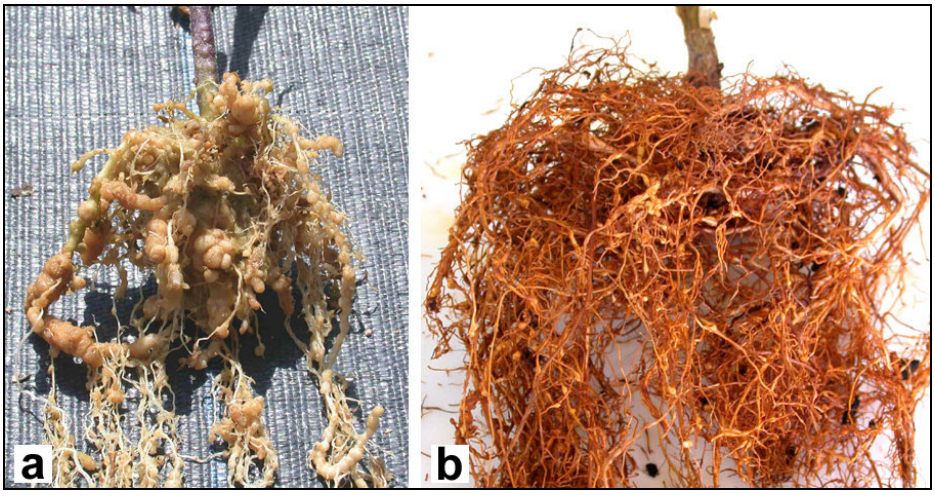

Figure 2.4. Roots of tomato (a) and GF-677 rootstock (b) infected by the root-knot nematode *Meloidogyne javanica*.

Meloidogyne *spp. root-knot on rootstocks*

A rootstock is a stump with an already established root system that interacts with the soil to nourish the aerial part (a cutting from another tree plant or scion). The scion is grafted with the rootstock and eventually the tissues of the two parts will fuse and will produce a single tree,

although both parts will remain genetically different. The use of rootstocks is most commonly associated with fruit trees and is the only way to propagate many types of plants that do not breed from seed or are particularly susceptible to soil-borne pathogens when they grow on their own roots.

The emergence of problems derived from repeated plantings of fruit trees in Spain as a consequence of the increase of the fruit production industry and a progressive decrease of soil quality due to continuous planting and an inoculum increase of soil-borne pathogens has lead to the replacement of traditional rootstocks with new plant material, which is vegetativelly propagated and possess superior agronomic characteristics. There is an urging demand for the evaluation of the susceptibility against soil-borne pathogens, especially fungi and nematodes, of new rootstock plant material (Pinochet, 1997; Felipe *et al.*, 1998).

Soil-borne pathogens such as nematodes and fungi are the main causal agents of replanting problems. In Spain, root-knot nematodes (*Meloidogyne* spp.) are frequently associated with rootstock diseases. Some rootstocks are resistant to *M. javanica*, *M. incognita* and *M. arenaria* (e.g. apricot, cherry and the plum rootstocks Julian 655-2, Marianna 2624, among others). The rootstock GF-677 is a natural hydrid of peach and almond (*Prunus persica x Prunus dulcis*) originated in France (INRA, Bordeaux, France) and one of the most commercially important in Spain and western Europe. This rootstock is better adapted to well-drained soils and, even though is tolerant to chlorosis, is highly susceptible to root-knot nematodes (*Meloidogyne* spp.) (Fernández *et al.*, 1994) and susceptible to lesion nematodes (*Pratylenchus* spp.) (Pinochet *et al.*, 2000) and other soil-borne pathogens such as *Agrobacterium* spp., *Armillaria* spp. and *Phytophthora* spp. (Felipe *et al.*, 1998).

Control methods

The real impact of nematodes on yield is difficult to establish accurately because many of the effective control strategies, such as soil fumigants, also target other soil-borne pathogens (Bird and Kaloshian, 2003). Yield losses by nematodes ranges from 8% to 20% annually on major crops around the world (Bird and Kaloshian, 2003; Bird *et al.*, 2003; Dong and Zhang, 2006). Among all control methods used against nematodes, chemical control is the most widely used. In some regions such as southern Europe, the Mediterranean region and North Africa, many vegetable crops are commonly treated with soil fumigants and non-fumigant nematicides, with a high economical cost. Because of environmental issues, high costs, residues on food, animal

toxicity (nematicides are highly toxic compounds with very low LD_{50} values), and progressive reduction of effectiveness after repeated applications, chemical fumigants (e.g. methyl bromide, 1,3-dichloropropene, ethylene dibromide, metam sodium...) and nematicides (e.g. organophosphates and carbamates) are being restricted or have been withdrawn from the market in most countries (Gowen, 1997; Dong and Zhang, 2006). Integrated pest management (IPM) permits to combine less hazardous chemical nematicides with other alternative control methods used rationally in sustainable agricultural systems; for example, solarization, cover crops, green manures, organic or inorganic soil amendments, crop rotations, use of resistant or tolerant cultivars and biological control (Oka *et al.*, 2000; Dong and Zhang, 2006).

Biological control of plant-parasitic nematodes

Biological control can be defined as the use of beneficial microorganisms to control plant pathogens. Beneficial microorganisms with the ability to control plant-parasitic nematodes include nematophagous fungi, endophytic fungi, actinomycetes and bacteria, especially rhizobacteria. Some of these microorganisms have been commercialized, especially nematophagous fungal species and bacteria (Table 1, pages 6-7) (Dong and Zhang, 2006).

Microorganisms that can attack and reduce nematode populations can be divided into two groups. The first group is comprised by specialized antagonists that are able to control nematodes via production of toxins or via parasitism by means of infection structures such as adhesive spores or traps. Nematode parasites are the most studied biological control agents (BCA) of nematodes, and comprise bacteria, such as the actinomycete *Pasteuria penetrans*, and nematode-trapping fungi, such as the egg-parasite *Paecilomyces lilanicus*. The second group are non-specialized and non-parasitic antagonistic rhizobacteria, such as *Pseudomonas* spp., *Serratia* spp. or *Burkholderia* spp.. The mechanisms used by antagonistic rhizobacteria are often difficult to determine because they can be affected by multiple factors, especially in field conditions, such as environmental and edaphic factors, nematode species, and the developmental stage or physiological and genetic characteristics of the host plant. The mechanisms involved in biological control of nematodes by rhizobacteria can affect root-gall development, egg hatching, juvenile motility and nematode survival directly by production of toxic metabolites (e.g. ammonia, butyric acid, hydrogen sulphide, and other toxic compounds), degradation of specific root exudates and nematode attractants, or indirectly by alteration of root exudates or induction of systemic resistance (ISR or SAR) in the plant (Siddiqui and Mahmood, 1999; Kerry, 2000). In most cases, the biocontrol mechanism is production of toxic metabolites or direct contact

between the biocontrol microorganism and the nematode, although induction of systemic resistance has also been reported to control *Meloidogyne* spp. on tomato plants treated with *Pseudomonas* spp. (Siddiqui and Shaukat, 2002a; Siddiqui and Shaukat, 2004). Induced resistance is an interesting mechanism of nematode control because of its potential to be a broad-spectrum mechanism and its durability. Induced resistance reaches its maximum effectiveness four or five days after the application of the inducing agent, and its persistence, even though it generally decreases over time, ranges from 15 to 90 days, depending on the crop. Additionally, root or seed applications of a BCA that induce systemic resistance usually produce a longer effect than foliar applications (Ramamoorthy *et al.*, 2001).

Several fungal and bacterial species have been reported as biocontrol agents of nematodes on different pathosystems. Examples of nematode biocontrol have been reported in literature for different species of plant-parasitic nematodes: biocontrol of the root-lesion nematode *Pratylenchus vulnus* by *Glomus intraradices* on quince BA-29 rootstock (Calvet *et al.*, 1995), *Pratylenchus penetrans* by *Glomus* spp. on apple rootstocks (Forge *et al.*, 2001) and by *Streptomyces* spp. on alfalfa (Samac and Kinkel, 2001), biocontrol of the burrowing nematode *Radopholus similis* by *Pseudomonas* spp. on banana plants (Aalten *et al.*, 1998), or the cyst nematode *Globodera pallida* by *Rhizobium etli* on potato (Reitz *et al.*, 2000). According to several reports, biocontrol of *Meloidogyne* spp. by different beneficial microorganisms has been described in various pathosystems: biocontrol by the fungi *Paecilomyces lilacinus* (Kiewnick and Sikora, 2006) and *Trichoderma* spp. (Sharon *et al.*, 2001; Stevens *et al.*, 2003) on tomato, and by the bacteria *Bacillus* spp. on rice (Padgham and Sikora, 2006), and *Pseudomonas* spp. (applied individually or combined with other biocontrol bacteria or fungi) on tomato (Aalten *et al.*, 1998; Jonathan *et al.*, 2000; Siddiqui *et al.*, 2000; Siddiqui and Ehteshamul-Haque, 2000a; Siddiqui and Ehteshamul-Haque, 2000b; Siddiqui and Ehteshamul-Haque, 2001; Siddiqui *et al.*, 2001; Siddiqui *et al.*, 2003; Siddiqui and Shaukat, 2003), cucumber (Roberts *et al.*, 2005), banana plant (Aalten *et al.*, 1998; Jonathan *et al.*, 2000), chickpea (Siddiqui and Mahmood, 2001), and mung-bean (Ali *et al.*, 2002).

Control efficacy of these BCAs depends on multiple factors, especially in field conditions, such as the host plant (herbaceous or ligneous), and are only effective against specific stages of the nematode in a short period of time. In the specific case of fruit tree rootstocks, it is very important to find a biological control agent that, applied to the roots of the rootstock plantlets in the first stages of acclimatization when plantlets pass from *in vitro* to greenhouse conditions, could protect the plantlet from nematode infection or reduce its incidence so that healthier plants could be moved from nurseries to commercial fields. These

commercial fields should be previously disinfested by means of solarization or fumigation with less hazardous chemical products.

Objectives

The main aim of the study in this chapter was to find effective bacterial strains against *M. javanica* on the GF-677 rootstock. Putative biocontrol strains of *Pseudomonas fluorescens* and *Pantoea agglomerans* (syn. *Erwinia herbicola*) were selected from *in planta* screenings and for their metabolic and *in vitro* antagonistic activities and characterized. Combinations of the best strains were also tested in plants and their effect in biocontrol was compared to the effect of strains applied individually.

Biological Control of *Meloidogyne javanica* in GF-677 Rootstocks with *Pseudomonas fluorescens* Strains and their Combination

Abstract

Pseudomonas fluorescens strains EPS384, EPS895 and CHA0 were selected from a collection of 58 *P. fluorescens* and *Pantoea agglomerans* strains for their efficacy in reduction of *Meloidogyne javanica* infection in GF-677 rootstocks, as well as for their *in vitro* antagonism and metabolic activity, by principal components analysis. EPS384 synthesized salicylic acid (SA), siderophores, and solubilized inorganic phosphates, whereas EPS895 synthesized pyoluteorin and siderophores. Strain CHA0 presented a high metabolic activity, synthesizing siderophores, 1-indole-3-acetic acid, hydrogen cyanide, 2,4-diacetylphloroglucinol, pyrrolnitrin, and SA, and showing inorganic phosphate solubilization and chitinolytic activities. All three bacterial strains significantly reduced juvenile survival *in vitro*, and strains EPS384 and CHA0 also significantly reduced *M. javanica* egg hatching *in vitro*. Additionally, EPS384 significantly reduced root galling in a split-root system, suggesting that the mechanism of action of this strain was induction of plant resistance. Individual and combined applications of strains EPS384 and EPS895 produced a significative reduction of *M. javanica* infection on rootstock plants. The combination of strains reduced variability between experiments, thus increasing consistency, but biocontrol efficacy was maintained compared to individual applications.

Keywords: Peach x almond hybrid, root-knot nematode, *Pseudomonas fluorescens*, *Pantoea agglomerans*, biological control.

Introduction

The emergence of problems derived from repeated plantings of fruit trees in Spain is a consequence of the increase of the fruit production industry and a progressive decrease of soil quality due to continuous planting and soil-borne pathogens (Calvet *et al.*, 2000), and has lead to the replacement of traditional rootstocks with new plant material vegetativelly propagated that possess superior agronomic characteristics. The rootstock GF-677 is a natural hydrid of peach and almond (*Prunus persica* x *Prunus dulcis*) originated in France (INRA, Bordeaux, France) and one of the most commercially important in Spain. This rootstock is better adapted to well-drained soils and, even though is tolerant to chlorosis, is susceptible to nematodes, especially to root-knot nematodes (*Meloidogyne* spp.) (Fernández *et al.*, 1994; Felipe *et al.*, 1998). *Meloidogyne* species are the most damaging root-knot nematodes. These nematodes are distributed worldwide, although the most important species, such as *M. javanica*, *M. incognita* and *M. arenaria*, are more adapted to warm and tropical climates (Pinochet and Bello, 2000).

The real impact of nematodes on yield is difficult to establish accurately because many of the effective control strategies, such as soil fumigants, also target other soil-borne pathogens (Bird and Kaloshian, 2003). Yield losses by nematodes ranges from 8% to 20% annually on major crops around the world (Bird and Kaloshian, 2003; Bird *et al.*, 2003; Dong and Zhang, 2006). Among all control methods used against nematodes, chemical

control is the most widely used. However, because of environmental and health issues, the use of chemical nematicides is currently being more regulated and restricted in most countries (Dong and Zhang, 2006). Integrated pest management permit to combine less hazardous chemical nematicides with other alternative control methods used rationally in sustainable agricultural systems, for example biological control (Dong and Zhang, 2006). A biocontrol agent applied to the roots of the rootstock plantlets in the first stages of acclimatization could protect the plantlet from nematode infection or reduce its incidence so that healthier plants could be moved from nurseries to commercial fields, which would be previously disinfested by means of solarization or fumigation with less hazardous chemical products.

Beneficial microorganisms with the ability to control plant-parasitic nematodes include nematophagous fungi, endophytic and mycorrhizal fungi, actinomycetes and bacteria, especially rhizobacteria. Some of these microorganisms have been commercialized, especially nematophagous fungal species and bacteria (Dong and Zhang, 2006).

Several fungal and bacterial species have been reported as biocontrol agents of nematodes on different pathosystems. Examples of biological control of *Meloidogyne* spp. have been reported on herbaceous plants. For instance, biocontrol by the fungi *Paecilomyces lilacinus* (Kiewnick and Sikora, 2006) and *Trichoderma* spp. (Sharon *et al.*, 2001; Stevens *et al.*, 2003) on tomato, by *Bacillus* spp on rice (Padgham and Sikora, 2006), and by *Pseudomonas* spp. applied individually or combined with other biocontrol bacteria or fungi on tomato (Aalten *et al.*, 1998; Jonathan *et al.*, 2000; Siddiqui *et al.*, 2000; Siddiqui and Ehteshamul-Haque, 2000a; Siddiqui and Ehteshamul-Haque, 2000b; Siddiqui and Ehteshamul-Haque, 2001; Siddiqui *et al.*, 2001; Siddiqui *et al.*, 2003; Siddiqui and Shaukat, 2003), cucumber (Roberts *et al.*, 2005), chickpea (Siddiqui and Mahmood, 2001), mung-bean (Ali *et al.*, 2002) and banana plants (Jaizme-Vega *et al.*, 1997; Aalten *et al.*, 1998; Jonathan *et al.*, 2000).

Some examples of biocontrol in woody plants are biocontrol of the root-lesion nematode *Pratylenchus penetrans* by *Glomus* spp. on apple rootstocks (Forge *et al.*, 2001), biocontrol of *Pratylenchus vulnus* by *Glomus intraradices* on plum (Pinochet *et al.*, 1998), and of the migratory ectoparasitic ring nematode *Mesocriconema xenoplax* by *Pseudomonas* spp. on peach trees (Kluepfel *et al.*, 2002). Biocontrol of *Meloidogyne* spp. on rootstock trees is usually associated to arbuscular mycorrhizal fungi applied individually or in combination with rhizobacteria (Castillo *et al.*, 2006; Jaizme-Vega *et al.*, 2006).

Nevertheless, the efficacy of biological control is occasionally inadequate and variability in control efficacy may be high. For this reason, it is important to select biocontrol strains that efficiently reduce nematode infection by means of the stimulation of plant defenses and/or by combination with other biocontrol strains with different mechanisms of action. The mechanisms used by these beneficial microorganisms are often difficult to determine because they can be affected by multiple factors, especially in field conditions, such as environmental and edaphic factors, nematode species and developmental stage or physiological and genetic characteristics of the host plant. The mechanisms involved in biological control affect root-gall development, egg hatching or nematode survival directly by production of toxic metabolites, or indirectly by induction of systemic resistance in the plant. In most cases, the biocontrol mechanism consists of production of toxic metabolites or direct contact between the biocontrol microorganism and the nematode, although induction of systemic resistance has also proved to control *Meloidogyne* spp. on tomato plants treated with *Pseudomonas* spp. (Siddiqui

and Shaukat, 2002; Siddiqui and Shaukat, 2004).

The aim of the present study was to select effective bacterial strains against *M. javanica* on the GF-677 rootstock. *Pseudomonas fluorescens* strains that significantly suppressed root-knot disease were selected from a collection of putative biocontrol *P. fluorescens* and *Pantoea agglomerans* strains and characterized. Biocontrol mechanisms were determined and the efficacy of the combination of selected strains was tested on GF-677 and compared to applications of individual strains.

Materials and Methods

M. javanica inoculum

The population of the root-knot nematode *Meloidogyne javanica* (Treub) Chitwood used in this study was originally isolated from infested GF-677 in a peach orchard in San José de la Rinconada (Seville, Spain) and was cultivated on tomato roots of cultivar Rio Grande (Agromillora Catalana, Barcelona, Spain). The population was maintained in storage cv. Rio Grande tomato plants under greenhouse conditions and continually multiplied by transferring infested root material to new plant material every two or three months. Egg and juvenile suspensions were obtained by the Cobb's method (Shurtleff and Averre, 2000). Briefly, 2-5 g of tomato roots infested with galls were washed to remove sand and substrate particles, diced into 2- to 3-cm long fragments, and placed in an electric blender with 200 ml of tap water with sodium hypochlorite (2% active chlorine). After grinding the plant material for 30 s to avoid damaging the nematodes, the mixture was poured through a 200-Mesh sieve (75 μm pore size) nested on top of a 500-Mesh sieve (25 μm pore size) and thoroughly washed. The 500-Mesh sieve was rinsed to remove the nematodes retained on it, that were collected into a 200-ml beaker. The volume of suspension collected was measured and egg and nematode concentration was assessed with a counting chamber at 40X (Olympic Equine Products, Issaquah, WA, USA). *M. javanica* suspensions were prepared immediately before assays.

Disinfection of *M. javanica* egg suspension was performed in the *in vitro* assay by adding sodium hypochlorite (3% active chlorine) to the suspension and gently agitating it for 20 min. These conditions allowed an optimal egg surface disinfection. The suspension was poured onto an autoclaved 500-Mesh sieve, and the retained sediment was rinsed with sterile distilled water to wash away traces of sodium hypochlorite. To collect the nematode suspension, the sieve surface was rinsed with sterile distilled water. The egg concentration was assessed as described above.

Bacterial strains and culture conditions

A total of 46 bacterial strains of *Pseudomonas fluorescens* and *Pantoea agglomerans* were obtained from the strain collection of the Institute of Food and Agricultural Technology (Girona, Spain) and selected for their biocontrol activities based upon preliminary laboratory assays. Additionally, 12 strains of *P. fluorescens* from culture collections reported in literature as biocontrol agents were included (Chapter I, page 58).

Bacteria were stored at -80ºC in 20% glycerol Luria-Bertani broth (LB) (Maniatis *et al.*, 1982), and were routinely cultured on LB agar at 23ºC prior to experiments. Bacterial suspensions were prepared from 24-h LB agar cultures grown at 23ºC, inoculated into 10 ml of LB broth in culture tubes, and incubated at 23ºC on a rotatory shaker at 150 rpm for 24 h. Cells were then pelleted by centrifugation at 8000 x *g* for 15 min, resuspended in sterile distilled water and the final concentration was adjusted to 10^9 cfu ml^{-1}.

Plant material

GF-677 rootstock plantlets were used in experiments. Plants were produced by *in vitro* micropropagation in a commercial nursery (Agromillora Catalana, Barcelona, Spain). Experiments were performed with acclimatized 15- to 30-day-old plantlets (6-10 cm height) provided in small minipot trays. Plantlets were introduced into 200-ml pots containing a sand:peat (3:1) mixture and maintained in greenhouse at 30 ± 5ºC and a 16-h photoperiod. Plants were transplanted into 1.5-liter pots containing a sand:peat (4:1) mixture 15 to 20 days after.

Strain characterization

The presence of biosynthetic genes for production of 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), pyrrolnitrin (Prn) and pyoluteorin (Plt) was determined by PCR using the primers and conditions previously described by Raaijmakers *et al.* (1997), Badosa (2001) and Mavrodi *et al.* (2001).

Production of salicylic acid (SA), PCA, DAPG, Prn and Plt in Luria-Bertani broth (LB) was assessed using high performance liquid chromatography (HPLC) and nuclear magnetic resonance (^{1}H-NMR) techniques. Liquid cultures of the strains were incubated on LB for 48 h at 25ºC and centrifuged at 8000 x *g* for 15 min. To extract metabolites for HPLC analysis, a 10-ml aliquot of each supernatant was acidified to pH 3.0 with 10% trichloroacetic acid and was extracted with a solid-phase hydrophobic adsorbant column (RESPREP SPE Cartridges: Bonded reversed phase, C_{18}, 3 ml syringe volume). The column was washed with 0.5 ml H_2O milliQ and metabolites were disadsorbed with a 1-ml solution of acetonitrile and 15% H_2O milliQ. Samples of 20 µl were injected in a Waters HPLC (model 610, Waters, Mildford, Madison, WI, USA) equiped with an analytical column (Resteck, Pinnacle II C_{18}, 5 µm, 150 x 4.6 mm) packed with Tracer Hypersil ODS attached to a precolumn (10 x 0.4 mm). The column was eluted with a solvent system of acetonitrile:water acidified at pH 2.5 with trichloroacetic acid (40:60, v/v), at a flow rate of 1 ml min^{-1}. Measurements were performed with a detector (model 484, Waters, Mildford, WI, USA) at λ=270 nm. Under these conditions, SA, PCA, DAPG, Plt and Prn were detected respectively at 4.43, 8.85, 9.73, 5.98 and 30.12 min retention time.

Metabolites extraction for NMR analysis was performed as follows. A 30-ml aliquot of each supernatant was acidified to pH 3.0 with 10% trichloroacetic acid and was extracted with a solid-phase hydrophobic adsorbant column (RESPREP SPE Cartridges: Bonded reversed phase, C_{18}, 3 ml syringe volume). The column was washed with 1.5 ml acetonitrile to desadsorb metabolites and the solution was reduced to dryness under vacuum. The dry residue was dissolved in D_6-acetone and analysed by nuclear magnetic resonance (^{1}H-NMR) at 200 MHz.

Hydrogen cyanide (HCN) production was assessed by the picrate method (Sneath, 1966). Tubes containing LB agar and a filter paper strip impregnated with 0.5% picric acid and 2% $NaCO_3$ introduced into the test tube screw cup were inoculated with the bacteria. Upon incubation at 22ºC for three days, production of cyanide was detected as a change of the filter paper colour from yellow to orange-brown.

1-indole-3-acetic acid (IAA) and related compounds were identified upon colony growth in LB agar amended with 5 mM l-tryptophan and overlaid with an 82-mm diameter nitrocellulose membrane disk (Brito Alvarez *et al.*, 1995). Agar plates were inoculated with bacterial cultures and incubated at 28ºC for three days. Then, the membranes were removed and placed overlaid on a Whatman filter paper impregnated with the Salkowski reagent. Strains producing IAA or analog substances were identified by a pink or red colour on the filter surface.

Siderophore production was determined using chrome azurol S (CAS) medium

(Schwyn and Neilands, 1987). CAS agar plates were inoculated with the strains and incubated at 28ºC for five days. Strains exhibiting an orange halo were positive for siderophore production.

Cellulase, mannanase, xylanase and β-1,3-glucanase production was tested on tryptone-soy agar (TSA) (Oxoid Ltd., Basingstoke, UK) amended with 1 mg ml^{-1} of specific chromogenic (azurine-dyed, cross-linked; AZCL) substrate (Megazyme, Sydney, Australia) for each enzyme test (Nielsen *et al.*, 1998). Bacterial colonies were transferred to the agar surface with sterile toothpicks. Agar plates were incubated at 23ºC for 10 days. Three replicates were made for each bacterial strain. Strains that produced homogenic blue zones were considered as positive for polymer hidrolysis activity.

Chitinolytic activity of bacterial strains was assessed using a chitin medium described by Frändberg and Schnürer (1997): 2.7 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.7 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 0.5 g of KCl, 0.13 g of $CaCl_2 \cdot H_2O$, 3 g of yeast extract, 1.5 g of colloidal chitin (Rodríguez-Kábana *et al.*, 1983) and 20 g of agar per liter. Colonies of the bacterial strains were transferred to the surface of the agar plates with sterile toothpicks. Three replicates for each bacterial strain were performed. Chitinolytic bacteria were identified as colonies surrounded by clearing zones after seven days of incubation at 23ºC.

Inorganic phosphate solubilization ability was assessed using three different media described by Nautiyal (1999) which contained $Ca_3(PO_4)_2$: Pikovskaya medium, NBRIY medium and NBRIP medium. Colonies of the bacterial strains were transferred to the surface of the agar plates with sterile toothpicks. Three replicates for each bacterial strain were performed. Strains that produced transparent zones after seven days of incubation at 23ºC were considered as positive in the phosphate solubilization ability test.

A RAPD-PCR analysis was performed to detect natural polymorphisms in the genome of *P. fluorescens* EPS384, EPS895 and CHA0. For DNA extraction, bacterial strains were grown in 1 ml of LB broth for 24 h at 25ºC. Then, cultures were centrifuged 2 min at 10000 x *g*, pellets were resuspended in 1 ml of sterile H_2O milliQ and centrifuged 2 min at 10000 x *g*. This step was repeated twice. Finally, pellets were resuspended in 150 µl of sterile H_2O milliQ, heat lysed (10 min at 99ºC) and centrifuged 1 min at 10000 x *g*. The extracted DNA was stored at -20ºC for later use. Amplification reactions were performed in a final volume of 25 µl containing 1X PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.4 µM of primer, 1.5 U of *Taq* DNA polymerase (Invitrogen, CA, USA) and 5 µl of the extracted DNA. The primer used was Phl2b (5'ACCGCAGC ATCGTGTATGAG3') (Raaijmakers *et al.*, 1997). RAPD fingerprints were obtained using the thermal cycle conditions described by Keel *et al.* (1996) with a minor modification: 2 cycles of 94ºC for 30 s, 36ºC for 30 s, and 72ºC for 120 s; 20 cycles of 94ºC for 20 s, 36ºC for 15 s, 45ºC for 15 s, and 72ºC for 90 s; 19 cycles of 94ºC for 30 s (incubation time increased 1 s per cycle), 36ºC for 15 s, 45ºC for 15 s, and 72ºC for 120 s (incubation time increased 3 s per cycle); and a final incubation at 72ºC for 10 min. The amplified products were visualized by gel electrophoresis in a 2% agarose gel with 1X TAE and stained with ethidium bromide.

M. javanica egg hatch and juvenile survival *in vitro* assay

Suspensions of *M. javanica* eggs and juveniles were incubated in liquid medium (LB broth) and in solid substrate (sterile sand) to test the effect of selected *P. fluorescens* strains EPS384, EPS895 and CHA0 on pathogen viability after six days of co-incubation. Since metabolite production by bacterial strains was assessed in LB broth, this medium was used in the *in vitro* test.

1. *Liquid medium* in vitro *test*

To prepare bacterial suspensions and cell-free bacterial culture extracts, 48-h bacterial cultures of *P. fluorescens* strains EPS384, EPS895 and CHA0 grown on LB broth were centrifuged at 4000 x *g* for 20 min. Supernatants (bacterial culture extracts) were separated from pellets and filtered with a 0.22-µm pore size filter membrane. Bacterial suspensions were prepared by resuspending the pellets in sterile distilled water and adjusting the final concentration to 10^8 cfu ml^{-1}. Two different treatments were performed. The first treatment consisted of the co-incubation of bacterial cells with *M. javanica* on sterile LB broth, whereas the second treatment consisted of the incubation of *M. javanica* on cell-free culture extracts from cultures of the bacterial strains grown on LB broth. To determine the nematicidal activity of bacterial suspensions and cell-free culture extracts, 100 µl of a surface-disinfected *M. javanica* suspension (8000 eggs per ml) were mixed with 100 µl of bacterial suspension in 1.8 ml of sterile medium or with 100 µl of sterile distilled water in 1.8 ml of cell-free culture extract. Non-treated controls consisted of *M. javanica* incubated on sterile LB broth. All treatments were performed in sterile 30-ml tubes and the final volume was 2 ml. Tubes were sealed with parafilm and incubated six days at 27ºC in darkness. To reduce anoxia, tubes were incubated inclined and were daily opened and gently shaken under sterille conditions. At the end of the assay, concentration of hatched and non-hatched eggs, as well as dead and viable juveniles (J2), were assessed with a counting chamber observed with an optical microscope at 40X. Immobile juveniles that appeared granular and non-translucent were considered as dead juveniles. For each treatment, three sets of one tube were performed. The experiment was repeated twice.

2. *Solid substrate* in vitro *test*

This experiment was performed in autoclaved sieved sand and *P. fluorescens* EPS384 and EPS895 were tested. Bacterial suspensions were prepared by centrifugating 24-h cultures of strains on LB broth at 4000 x *g* for 20 min, resuspending the pellets in sterile distilled water, and then adjusting the final concentration to 10^8 cfu ml^{-1}. 2 ml of a surface-disinfected *M. javanica* egg suspension (4000 eggs per ml), 1 ml of bacterial suspension and 1 ml of sterile distilled water were placed into sterile 30-ml tubes containing 20 g of autoclaved sieved sand. Non-treated controls consisted of the *M. javanica* egg suspension incubated in sterile 30-ml tubes containing 2 ml of sterile distilled water and 20 g of autoclaved sieved sand. Tubes were sealed with parafilm and incubated six days at 27ºC in darkness. At the end of the assay, nematodes were extracted by pouring the sand onto a 200-Mesh sieve nested on top of a 500-Mesh sieve and thoroughly washing the sand. The 500-Mesh sieve was rinsed and nematodes were collected, the volume of collected suspension was measured and concentrations of hatched and non-hatched eggs, as well as dead and viable juveniles (J2), were assessed with a counting chamber observed with an optical microscope at 40X. For each treatment, three sets of one tube were performed. The experiment was repeated twice.

Biological control of *M. javanica* on the GF-677 rootstock

GF-677 plantlets were treated with bacterial suspensions before and after being inoculated with a *M. javanica* suspension, which was prepared as previously described. Two experiments were performed. In first experiment, 58 bacterial strains (Chapter I, page 58) were tested and a total of six bacteria applications were performed, whereas in second experiment, 10 strains selected from first experiment were tested in a total of five bacteria applications.

In both experiments, first bacteria application was performed at the moment of planting by dipping the root system of 15-day-old rootstock plantlets in a bacterial

suspension adjusted to 10^9 cfu ml^{-1}. Bacteria-treated plantlets were introduced into 200-ml pots containing a sand:peat (3:1) mixture and maintained in greenhouse at 30 ± 5ºC and a 16-h photoperiod during the experiments. In first experiment, five additional bacterial treatments were performed 15, 28, 43, 57 and 105 days after first treatment by irrigation with 8 ml of 10^9 cfu ml^{-1} suspensions. *M. javanica* inoculation (6000 eggs per plant) was performed 34 days after first treatment. Plants were transplanted into 1.5-liter pots containing a sand:peat (4:1) mixture seven days before *M. javanica* inoculation. In second experiment, four additional bacteria applications were performed 20, 33, 53 and 66 days after first treatment by irrigation with 8 ml of 10^9 cfu ml^{-1} suspensions. *M. javanica* inoculation (2000 eggs per plant) was performed 36 days after first treatment. Plants were transplanted into 1000-ml pots containing a sand:peat (4:1) mixture 15 days before *M. javanica* inoculation. In both experiments, *M. javanica* egg suspension was inoculated by irrigation on potted plants. Non-treated control plants were irrigated with distilled water.

Plants were maintained in a greenhouse at 30 ± 5ºC, with a 16-h photoperiod. Total assay duration was 19 weeks in first experiment, and 18 weeks in second experiment. Growth parameters, such as shoot height, shoot fresh and dry weight, root fresh and dry weight, and shoot and root water content, as well as number of galls per root system and number of eggs per root system, were assessed at the end of the assay (14 weeks after pathogen inoculation in first experiment, and 13 weeks after pathogen inoculation in second experiment). In first experiment, five sets of one plant per treatment were arranged in a randomized experimental design, whereas second experiment consisted of three sets of five plants per treatment.

P. fluorescens EPS384, EPS895 and their combination (1:1) were evaluated for biological control of *M. javanica* in the GF-677 rootstock. GF-677 plantlets were treated with bacterial suspensions before and after being inoculated with a *M. javanica* suspension. In all experiments, a total of six bacteria applications were performed as described in the screening assay. First bacteria application was performed at the moment of planting and five additional bacterial treatments were performed 5, 12, 27, 35 and 58 days after first treatment by irrigation with 10 ml of 10^9 cfu ml^{-1} suspensions. *M. javanica* inoculation (2000 eggs per plant) was performed six days after first treatment. Non-treated control plants were irrigated with distilled water. The experiment was repeated twice, and the total duration of both experiments was nine weeks. Number of galls g^{-1} root were assessed at the end of the assay (69 days after pathogen inoculation and 12 days after sixth treatment). In both experiments, three sets of three plants per treatment were arranged in a randomized experimental design.

Assessment of population levels of inoculated bacterial strains on treated GF-677 *Prunus* rootstocks roots

The population levels of *P. fluorescens* EPS384 and EPS895 on treated plants were assessed. Spontaneous resistant mutant of strain EPS384 to 100 mg ml^{-1} of nalidixic acid but sensible to rifampicin, and spontaneous resistant mutant of strain EPS895 to 100 mg ml^{-1} of rifampicin but sensible to nalidixic acid were used in experiments. GF-677 plantlets were treated with suspensions of strains EPS384 and EPS895 applied individually or in combination, as described above in previous assays. First bacteria application was performed at the moment of planting by dipping the root system of 15-day-old rootstock plantlets in a bacterial suspension adjusted to 10^9 cfu ml^{-1}. Bacteria-treated plantlets were introduced into 500-ml pots containing a sand:peat (3:1) mixture and maintained in greenhouse at 30 ± 5ºC and a 16-h photoperiod during the experiments. Five additional bacterial treatments were

performed periodically after first treatment by irrigation with 8 ml of 10^9 cfu ml^{-1} suspensions. The experiment was repeated twice and, in both experiments, three sets of three plants per treatment were arranged in a randomized experimental design. To assess bacterial population levels, samples of plant roots were diluted in buffered peptone water (0.1% Bacto peptone in 30 mM potassium phosphate buffer 70 mM, pH 7.0), and extracted in a stomacher for 1 min. Suspensions were serially diluted in sterile distilled water. Appropiate dilutions were plated onto LB agar plates amended with 100 mg of rifampicin or nalidixic acid ml^{-1}, and incubated at 23ºC for 48 h. Colonies were counted and data were transformed to $\log_{10}$ cfu g^{-1} of fresh root weight.

Split-root test

A split-root test was performed to assess induction of plant resistance in the GF-677 rootstock by *P. fluorescens* EPS384. This split-root system allowed inoculation of the bacterial strain and the nematode at separate locations on the root system. Similar split-root tests had been conducted on other pathosystems as it had been reported in literature (Siddiqui and Shaukat, 2002). A three-pot system was developed to allow 15-day-old plantlets to grow half of their root system into two separated 8-cm diameter 500-ml pots filled with a sand:peat (3:1) mixture and attached together with staples (Figure 1).

Plants were allowed to grow for seven days before the first bacteria application. A total of five bacteria applications were performed 7, 14, 29, 42 and 65 days after planting and all bacterial treatments were applied on one of the pots by irrigation with 10 ml of a 10^9 cfu ml^{-1} suspension. 16 days after planting, the untreated half of the root system was infested with 2000 *M. javanica* eggs per plant. Non-treated control plants were irrigated with distilled water. The experiment was repeated twice, and the total duration of both experiments was 76 days. 60 days after *M. javanica* inoculation, root fresh weight, and total number of galls per half-root and galls g^{-1} root (the nematode-inoculated half root) were assessed. In both experiments, three sets of three plants per treatment were arranged in a randomized experimental design.

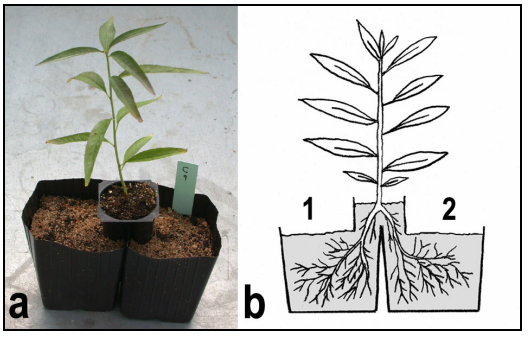

Figure 1. Split-root system used to test the effect of *P. fluorescens* EPS384 on *M. javanica* infection when applied spatially-separated on GF-677 rootstock plantlets. (a) Three-pot system used in the split-root test. (b) Diagram of the split-root system; *M. javanica* was inoculated in one compartment (1) and applications of EPS384 were performed in the other compartment (2).

Data analysis

Principal components analysis (PC) was performed using the PRINCOMP procedure of the SAS software. The principal components analysis was used to evaluate singularities among bacterial profiles to select the most suitable bacterial strains as *M. javanica* biocontrol agents (Figure 2). Principal components analysis was performed on four variables using 58 bacterial strains (Chapter I, page 58). The variables root galling, reproduction index (RI), metabolic activity and *in vitro* antagonism were calculated from results obtained in this study and in previous studies (Annex II). The variable root galling was the difference between the number of galls per root system of each treatment and the non-treated control. The variable reproduction index was the final number of eggs per root system divided by the number

of inoculated eggs. The variable metabolic activity was the summatory of the metabolic characteristics of all 58 bacterial strains, which consisted of qualitative results, being production (1) and no production (0). These metabolic characteristics comprised the ability to synthesize secondary metabolites and other molecules (HCN, IAA, siderophores, DAPG, PCA, SA, Plt and Prn) and the ability to hydrolize different polymers (chitin, cellulose, β-1,3-glucan, mannan and xylan) and solibilize inorganic phosphate. The variable *in vitro* antagonism was the summatory of the qualitative results observed when these 58 bacterial strains were tested for *in vitro* antagonistic activity against different plant pathogens (*Phytophthora cactorum*, *Erwinia amylovora*, *Stemphylium vesicarium*, *Pseudomonas syringae* and *Penicillium expansum*) on agar culture plates in different media, and accounted for positive *in vitro* antagonism (1) or negative *in vitro* antagonism (0) (Montesinos *et al*., 1996; Bonaterra, 1997; Francés, 2000; Badosa, 2001; Cabrefiga, 2004).

All data were analyzed by analysis of variance (ANOVA) and the treatment means were separated by using Fisher's protected or unprotected least significant difference (LSD) test or Duncan's multiple range test at $P \leq 0.05$. The coefficient of variation between experiments was calculated for all single treatments and their combination in the biocontrol assay. The analysis was performed with the GLM procedure of the SAS software (version 8.2, SAS Institute, Cary, NC, USA). Homogeneity of variances was determined with Barlett's test.

Results

Principal components analysis

Bacterial strains ordination by principal components analysis are shown in Figure 2. The first principal component (PC1) explained 35.1% of variation and was correlated with root galling (0.6814) and reproduction index (0.6310), so PC1 separated strains by their biocontrol effectiveness against *M. javanica* since high PC1 values corresponded to strains that enhanced root galling and egg production and low values corresponded to strains that reduced these parameters. The second principal component (PC2) explained 27.5% of variation and was correlated with *in vitro* antagonism (0.7980), and thus separated strains by their overall *in vitro* activity against several plant pathogens; high PC2 values corresponded to strains that inhibited several plant pathogens *in vitro*, whereas low PC2 values corresponded to strains that did not show *in vitro* antagonism. Finally, the third principal component (PC3) explained 25.3% of variation and was correlated with metabolic activity (0.8757), so high PC3 values corresponded to strains that produced several antibiotic metabolites and presented a high enzymatic and phosphate solubilization activities.

Bacterial strains were distributed according to their singularities in the biocontrol of *M. javanica* and their *in vitro* antagonistic activity or metabolic activity (Figure 2). Strains that possessed unconspicuous singularities were distributed in the center around PC1 values, between -1.0 and 1.0, whereas strains that possessed remarkable singularities were distributed far from these values and more dispersed.

The most effective strains in the biocontrol of *M. javanica* were EPS326, EPS384, EPS495, EPS607, EPS622, EPS895, and CHA0, having a PC1 value lower than -1.4 (Figure 2a). Strains CHA0 and EPS384 showed high *in vitro* antagonistic activity, whereas among strains with medium *in vitro* antagonistic activity were EPS895, EPS326, EPS495, EPS607 and EPS622). On the contrary, strains that showed very low biocontrol efficacy against *M. javanica* had PC1 values higher than +1.5 and were distributed to the right side.

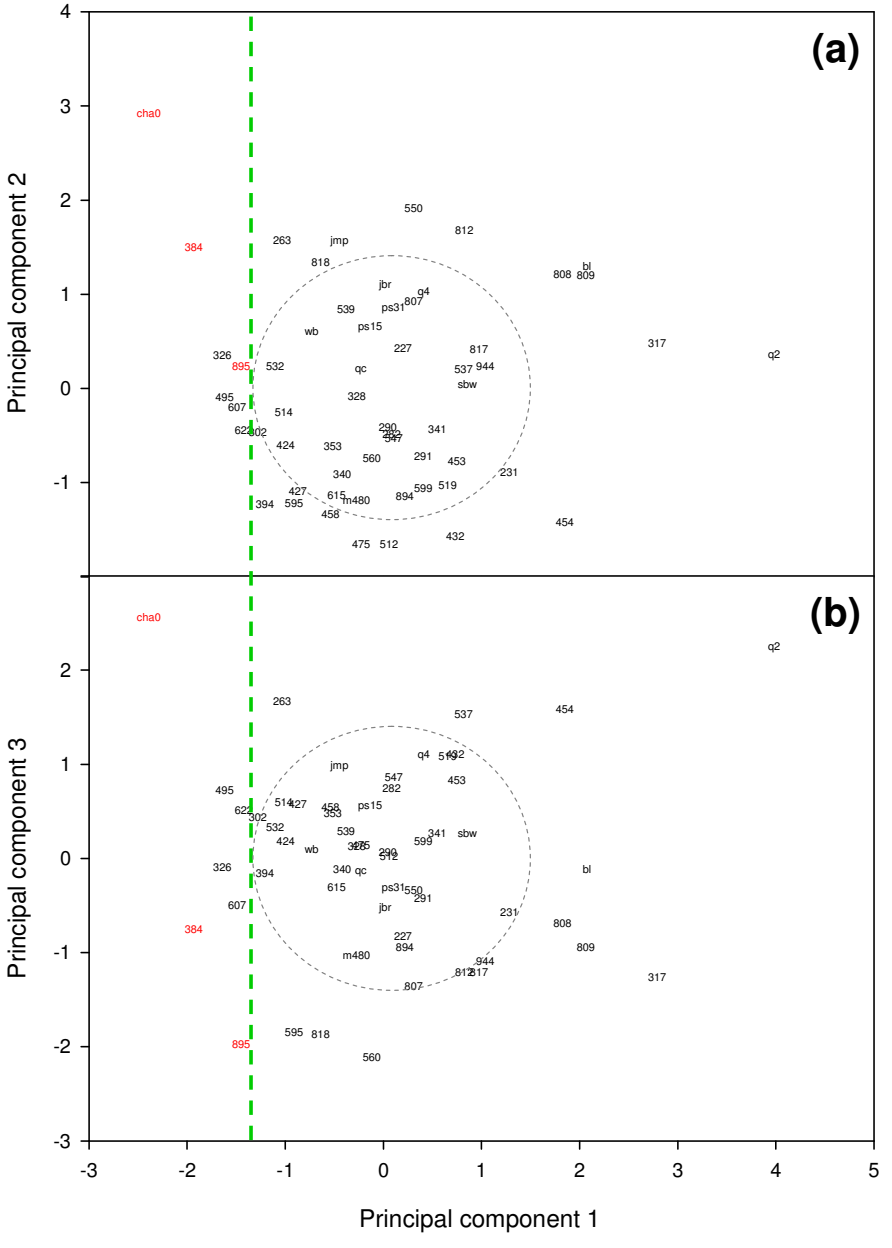

Figure 2. Principal components analysis of 58 *P. fluorescens* and *P. agglomerans* strains. (a) Scatter plot of bacterial strains on the first principal component (PC1) (*M. javanica* infection) and the second principal component (PC2) (*in vitro* antagonism). (b) Scatter plot of the PC1 (*M. javanica* infection) and the third principal component (PC3) (metabolic activity). Each code represents the values of the PC scores of each bacterial strain. The discontinued green line correspond to the PC1 value -1.4.

Dispersion of strains in the two dimensions consisting of PC1 and PC3 (metabolic activity) is showed in Figure 2b. Strains that highly reduced *M. javanica* infection and presented different levels of metabolic activity were distributed at PC1 values lower than -1.4. Among these strains, CHA0 showed high metabolic activity,

whereas strains EPS384, EPS326, EPS495, EPS607 and EPS622 showed medium levels and EPS895 showed low levels of metabolic activity. This PC analysis is a powerful tool that permitted to identify strains with high efficacy in reduction of *M. javanica* infection and different levels of metabolic activity. Selected *P. fluorescens* strains CHA0, EPS384 and EPS895 presented high, medium and low metabolic activity, respectively.

Strain characterization

P. fluorescens EPS384 synthesized siderophores, SA in LB, and showed inorganic phosphate solubilization ability (Table 1). *P. fluorescens* EPS895 synthesized siderophores and Plt in LB. Presence of genes related to PCA synthesis were detected by PCR, but synthesis of PCA by this strain was not detected in LB. *P. fluorescens* CHA0 synthesized Prn, SA, and DAPG in LB (production of monoacetylphloroglucinol (MAPG) was detected by HPLC at 20.95 min and was considered as a degradation product of DAPG). This strain also showed chitinolytic and inorganic phosphate solubilization abilities, and it was able to synthesize siderophores, IAA and HCN. Genes related to Plt synthesis were detected by PCR but production of this metabolite in LB was not detected by HPLC or NMR. None of these strains hydrolyzed any cell wall polymer tested.

The RAPD analysis showed different polymorphisms in all three bacterial strains and confirmed that they were genetically different (Figure 3).

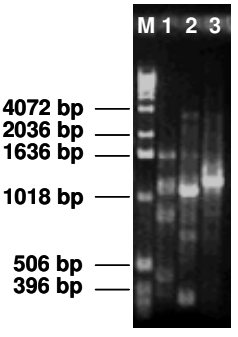

Figure 3. Genomic polymorphism patterns of selected *P. fluorescens* strains amplified by RAPD-PCR with primer Phl2b. (1) EPS384, (2) EPS895, (3) CHA0, (M) 1 kb DNA ladder marker (Invitrogen ™).

Table 1. Characterization of selected *Pseudomonas fluorescens* strains for production of molecules potentially related to biocontrol activity.

	Production of secondary metabolites and other molecules[v]									
	HCN	IAA	Sid	DAPG	PCA	SA	Prn	Plt	Chit[w]	Phos[x]
EPS384	-	-	+	-	-[z]	+	-	-	-	+
EPS895	-	-	+	-	-	-	-	+	-	-
CHA0	+	+	+	+[y]	-	+	+	+	+	+

[v] HCN, hydrogen cyanide; IAA, 1-indole-3-acetic acid; Sid, siderophores; DAPG, 2,4-diacetylphloroglucinol; PCA, phenazine-1-carboxilic acid; SA, salicylic acid; Prn, Pyrrolnitrin; Plt, Pyoluteorin. DAPG, PCA, Prn and Plt. Symbols correspond to presence of genes detected by PCR (Badosa, 2001) and metabolite detection by HPLC and NMR. SA symbols correspond to detection by HPLC and in SSM medium.

[w] Chitinolytic activity.

[x] Results are the overall of the inorganic phosphate solubilization activity on three media tested (Pikovskaya, NBRIY and NBRIP).

[y] MAPG was detected.

[z] PCA-related genes were detected by PCR, but not synthesis of PCA in LB.

Inhibition of *M. javanica* viability *in vitro*

The global ANOVA for the liquid media *in vitro* test showed that the effect of the incubation treatment (eggs incubated with bacterial cells or with cell-free extracts from 48-h bacterial cultures) was not significant, neither on egg hatching (P=0.6539 in experiment 1, and P=0.6795 in experiment 2) nor on juvenile survival (P=1.0000 in both experiments). On the other hand, the effect of bacterial strains was significant in experiments 1 and 2, either on egg hatching (P=0.0007 and P<0.0001, respectively) and on juvenile survival (P=0.0422 and P<0.0001, respectively).

A Duncan's multiple range test was performed to assess significant mean differences among different treatments and the non-treated control for each bacterial strain in both experiments (Figure 4). For all bacterial strains, the effect of the co-incubation of bacterial cells and *M. javanica* on egg hatching and on juvenile survival was not significantly different from the effect of the incubation of the nematode on cell-free culture extracts in both experiments. Therefore, *M. javanica* egg hatching and juvenile survival was equally affected by bacterial cells and cell-free culture extracts.

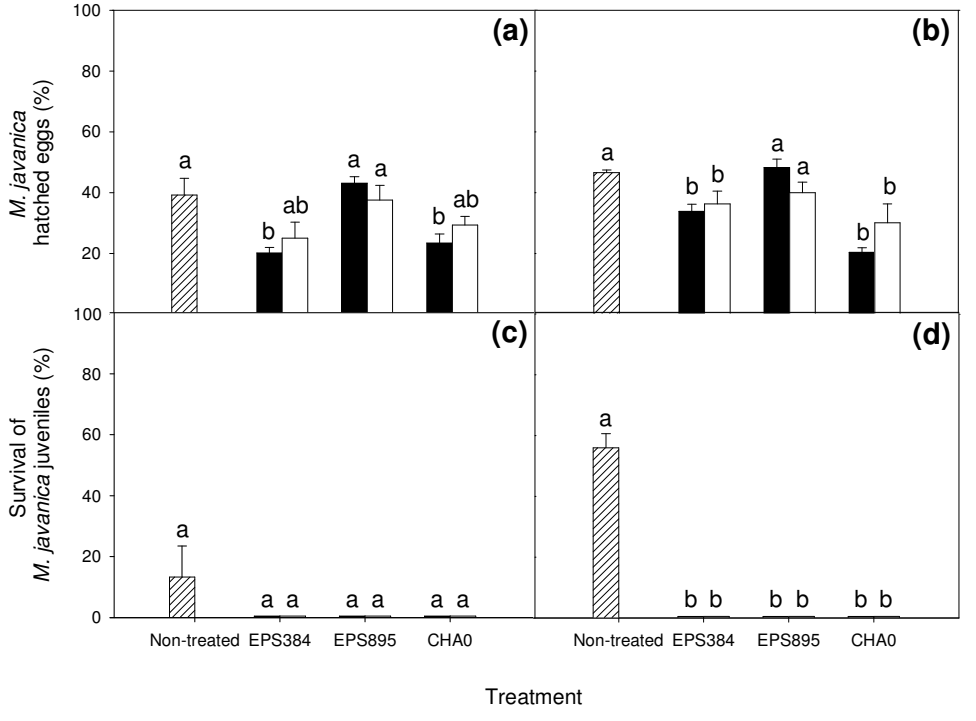

Figure 4. Effect of bacterial cell suspensions (black bars) and cell-free culture extracts (white bars) of *P. fluorescens* EPS384, EPS895 and CHA0 on egg hatching and second-stage juvenile (J2) survival of *M. javanica* six days after incubation at 27ºC in LB broth. Non-treated controls are represented by patterned bars. Values correspond to experiment 1 (a and c) and experiment 2 (b and d), and are the mean of three replicates. Error bars represent the mean standard error. Bars headed by different letters are significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

By comparing the effect of each bacterial strain on *M. javanica* egg hatching and juvenile survival with the non-treated control, some differences among strains could be observed. In both experiments, egg hatching was significantly reduced ($P<0.0500$) when eggs were co-incubated with bacterial cells and cell-free culture extracts of strains EPS384 and CHA0, but not with cells or cell-free extracts of strain EPS895, compared to the non-treated control. Reduction of egg hatching by strain EPS384 was 48.6% and 27.1%, and reduction by CHA0 was 40.2% and 56.3% in experiments 1 and 2, respectively, compared to the non-treated control. Incubation of *M. javanica* with bacterial cells or cell-free culture extracts only significantly reduced juvenile survival in experiment 2 ($P<0.0001$), whereas in experiment 1 bacterial cells and cell-free culture extracts did not have a significant effect ($P=0.2400$), although juvenile mortality reached 100% in both experiments.

In the solid substrate *in vitro* test, egg hatching and juvenile survival were not significantly affected ($P=0.3901$ and $P=0.0707$, respectively) by strains EPS384 and EPS895 when incubated on sterile sand in both experiments. In experiment 1, percentages of hatched eggs and juvenile survival in the non-treated control were 31% and 66%, respectively, whereas in experiment 2 these percentages were 39% and 72%, respectively.

Biological control of *M. javanica* on the GF-677 rootstock

In the first experiment, in which 58 bacterial strains were tested, non-treated control plants reached a mean of 609 galls per root system. The global ANOVA showed a significant effect ($P=0.004$) of bacterial treatment on total number of galls per root system of plants inoculated with *M. javanica* but very few strains reduced galls per root system compared to the non-treated control. Non-treated control plants reached 94317 eggs per root system (RI=15.7), but no significant effect ($P=0.1706$) of bacterial treatment was observed on total number of eggs per root system. Galls per root system and number of eggs per root system observed in infected plants treated with *P. fluorescens* EPS384, EPS895 and CHA0 are showed in Figure 5. Applications of these strains did not have a significant effect on number of galls per root system caused by *M. javanica* ($P=0.1001$) compared to the non-treated control. *M. javanica*-infected plants treated with these strains reached a significantly inferior number of eggs per root system ($P=0.0027$) compared to the non-treated control.

In the second experiment, where 10 of the 58 bacterial strains were tested, bacterial treatments had a significant effect ($P=0.0076$) on total number of galls per root system. Non-treated control plants reached 283.3 galls per root system, whereas strains EPS384, EPS895, EPS263, EPS326, EPS495, EPS607, EPS818, CHA0 and Q2-87 produced lower galling. Bacterial treatments significantly decreased ($P<0.0001$) the total number of eggs per root system, which was approximately 50.4% lower than the 47817 eggs per root system observed on non-treated control plants (RI=23.9). *P. fluorescens* EPS384, EPS895 and CHA0 significantly reduced root galling ($P=0.0126$) and number of eggs per root system ($P=0.0001$) compared to the non-treated control (Figure 5).

As for growth promotion of the GF-677 rootstock by treatment with bacterial strains, in the first experiment there were no significant differences between the non-treated control and treatments with strains EPS384, EPS895 and CHA0 in any growth parameter (shoot height, shoot fresh and dry weight, root fresh and dry weight, and shoot and root water content). In the second experiment, strains EPS384 and EPS895 did not have a significant effect on plant growth compared to the non-treated control. Strain CHA0 significantly increased shoot height

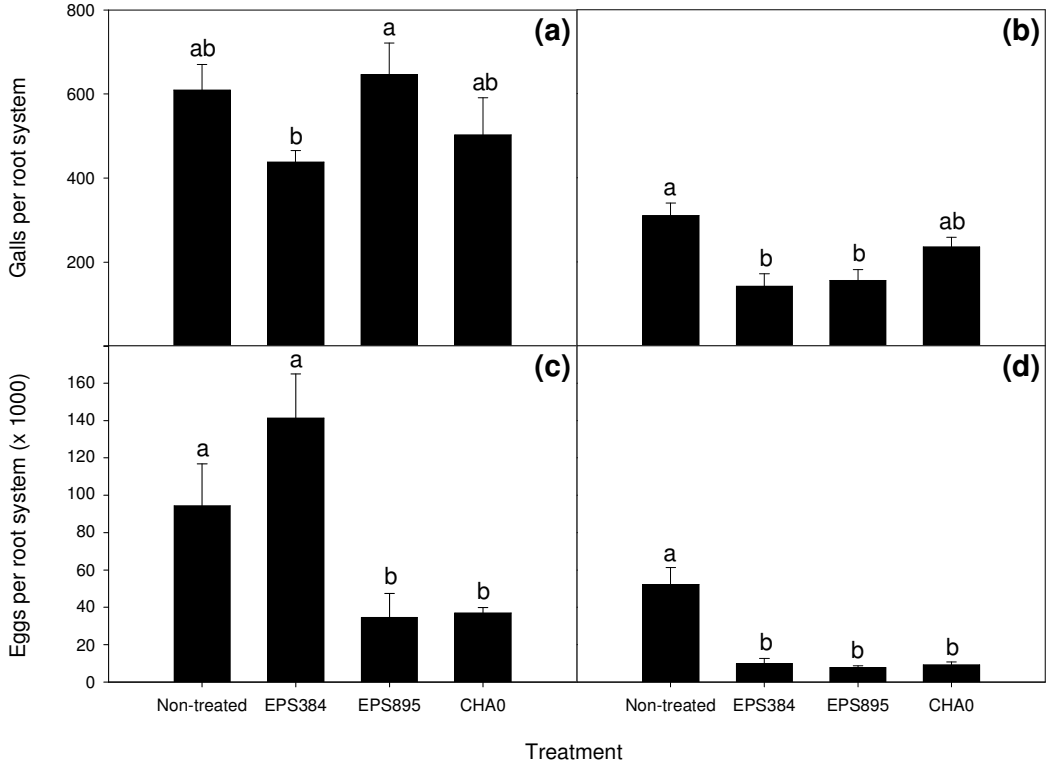

Figure 5. Effect of *P. fluorescens* EPS384, EPS895 and CHA0 on reduction of number of galls and eggs per root system on GF-677 rootstocks inoculated with *M. javanica*. Values correspond to the mean of five sets of one plant per treatment in experiment 1 (a and c), and five sets of three plants per treatment in experiment 2 (b and d). Error bars represent the mean standard error. Bars headed by different letters are significantly different ($P \leq 0.05$) according to Fisher's unprotected least significant (LSD) test.

(P=0.0003) and shoot water content (P=0.0041) of treated plants compared to non-treated control plants.

P. fluorescens EPS384, EPS895 and their combination were evaluated for biocontrol of *M. javanica* in the GF-677 rootstock. In the two independent experiments conducted, all treatments significantly reduced (P<0.0001) number of galls g^{-1} root in plants nine weeks after being inoculated with *M. javanica* (Figure 6). Strain EPS384 reduced number of galls g^{-1} root 68.9% (experiments 1) and 48.9% (experiment 2), compared to the non-treated control. Reduction of number of galls g^{-1} root by strain EPS895 was higher, being 77.2% and 58.4% in experiments 1 and 2, respectively. The combination of strains EPS384 and EPS895 did not improve biocontrol of *M. javanica* compared to individual treatments, since it produced a reduction of number of galls g^{-1} root of 43.3% (experiment 1) and 51.0% (experiment 2). In contrast, the combination of strains reduced variability between exp-eriments, resulting in a more consistent biocontrol efficacy.

In first experiment, reduction of *M. javanica* infection by the combination of strains EPS384 and EPS895 was not significantly different to the individual application of EPS384, but was significantly lower than the

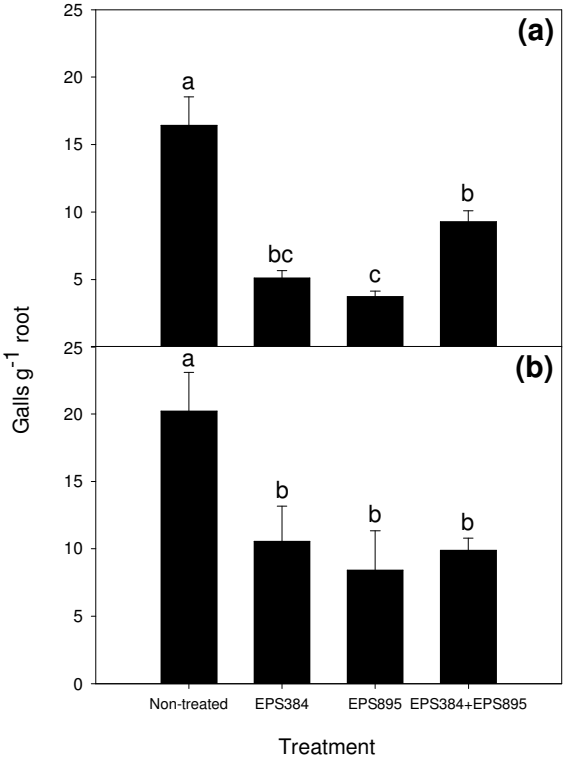

Figure 6. Effect of individual and combined bacterial treatments on gall formation on GF-677 rootstocks inoculated with *M. javanica* in two independent experiments (a and b). Values are the mean of three sets of three plants per treatment. Error bars represent the mean standard error. Bars headed by different letters are significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

reduction produced by application of EPS895 alone. In second experiment, all treatments produced a significantly similar reduction of *M. javanica* infection. The coefficient of variation between experiments was compared for each bacterial treatment in order to determine the variability of biocontrol of each treatment existing between experiments. The coefficient of variation between experiments corresponding to the non-treated control was 19.8%. The coefficient of variation between experiments of individual application of strains was 47.3% for EPS384 and 73.1% for EPS895. The combination of strains EPS384 and EPS895 produced a coefficient of variation between experiments lower compared to individual treatments, being 27.3%.

Population levels of inoculated bacterial strains on treated GF-677 rootstock roots

Roots treated with *P. fluorescens* EPS384 reached a population of $6.9x10^5$-$6.5x10^5$ cfu g^{-1} root in two independent experiments, while roots of plants treated with *P. fluorescens* EPS895 reached a population of $9.6x10^5$-$3.4x10^5$ cfu g^{-1} root in both experiments. The population levels of each strain in plants treated with a 1:1 mixture of EPS384 and EPS895 was of $6.2x10^5$-$8.2x10^5$ cfu g^{-1} root for EPS384 and of $1.5x10^5$-$2.4x10^5$ cfu g^{-1} root for EPS895, in both experiments. The global ANOVA did not show a significant effect of the experiment (P=0.1946), but there were significant differences among treatments (P=0.0004). Population levels reached by strains EPS384 and EPS895 applied individually were not significatively different (P>0.05) in both experiments.

However, when the two strains were applied in combination, population levels of EPS895 were significantly inferior (P=0.0122 and P=0.0315 in experiment 1 and 2, respectively) than population levels of EPS384.

Split-root test

Bacterial strain EPS384 and *M. javanica* were applied spatially separated in a split-root system to prevent direct contact. EPS384 applied to one half of the root system was not isolated from the non-bacteria treated nematode-inoculated half of the split-root system. Plant roots equally grew in both containers and no presence of the nematode or any gall formation was detected in the half root system in which the bacterial strain was applied or in the non-inoculated root system of non-treated control plants. Thus, no bacterial and nematode contamination existed from one container to the other. No differences on plant growth were observed through the experiment. Application of strain EPS384 to one half of the split-root system significantly reduced number of galls g^{-1} root (P=0.0018) in both experiments, being this reduction a mean of 42.8%, compared to the non-treated control (Figure 7).

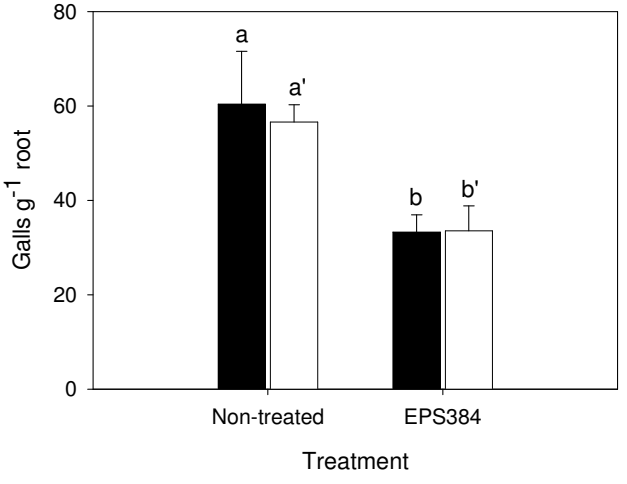

Figure 7. Effect of *P. fluorescens* EPS384 on *M. javanica* infection when applied spatially separated from the nematode on GF-677 rootstocks in a split-root system. Values correspond to experiment 1 (black bars) and experiment 2 (white bars), and are the mean of three sets of three plants per treatment. Error bars represent the mean standard error. Bars headed by different letters are significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Discussion

Three *Pseudomonas fluorescens* strains effective against *Meloidogyne javanica* on GF-677 were selected from a collection of 58 strains by means of a principal components analysis using variables based on reduction of *M. javanica* infection on GF-677, the metabolic activity and the *in vitro* antagonistic activity of bacterial strains. The principal components analysis provided a way to uncover trends of bacterial strains and permitted to identify these strains by their singularities based on their metabolic activity and *in vitro* antagonism. This analysis is a powerful tool to simplify information when several variables are used, since related variables can be explained by one principal component. In this study, two variables (gall formation and reproduction index) were simplified into the first principal component (PC1), which could be explained like a new variable (*M. javanica* infection).

Selected strains were *P. fluorescens* EPS384, EPS895 and CHA0. Strain EPS384 reduced infection and showed a moderate metabolic activity and high *in vitro* antagonism. Strain EPS895 showed low metabolic activity and *in vitro* antagonism, and high efficacy in *M. javanica* biocontrol. Finally, strain CHA0 showed high metabolic activity and *in vitro* antagonism, and also reduced *M. javanica* infection. Strain CHA0 was also selected as a reference biocontrol agent because of the extense literature reporting biocontrol of different soil-borne pathogens by this strain, including biocontrol of *M. javanica* on tomato (Siddiqui and Shaukat, 2002; Siddiqui and Shaukat, 2003) and oomycetal and fungal pathogens such as *Pythium* spp. on pea (Naseby *et al.*, 2001) and *Colletotrichum falcatum* on sugarcane (Senthil *et al.*, 2003). Additionally, these strains did not show a significant plant growth promotion effect, and only plants treated with CHA0 showed a rather significant increase on shoot growth compared to non-treated control plants. In fact, plant growth promotion by CHA0 has been previously reported in other plant species, for example in the plum rootstock Marianna 2624 (Bonaterra *et al.*, 2003) or in herbaceous plants such as pea (Naseby *et al.*, 2001) and sugarcane (Senthil *et al.*, 2003). In this study, selection of strains that do not promote plant growth was preferable since this plant growth-promoting effect would interfere with the inhibitory effect of strains on reduction of *M. javanica* infection. Also, the short experiment duration did not allow to determine growth promotion aptitudes more accurately.

Selected *P. fluorescens* strains EPS384, EPS895 and CHA0 were futherly characterized. Strain EPS384 synthesized siderophores and SA, and also showed inorganic phosphate solubilization ability, maybe via pH reduction by secretion of SA in the medium. This strain also reduced *M. javanica* egg hatching and produced high levels of juveniles mortality in LB broth, probably by pH reduction due to SA production or by anoxia. In the split-root assay, this strain significantly reduced gall formation when applied spatially-separated from *M. javanica*, and apparently induced plant resistance. The total number of eggs per root system was not assessed in this assay since differences in root galling between bacteria-treated plants and non-treated plants could be observed at the end of the experiment. This first study indicated that EPS384 may control *M. javanica* by means of induction of plant resistance, but further studies would determine whether this is the mechanism by which EPS384 reduces *M. javanica* infection in GF-677 rootstocks. Also, it would be interesting to assess the role of SA in the induction of resistance. Park and Kloepper (2000) observed that bacterial strains that produced SA induced PR proteins ex-pression, and therefore systemic resistance via SAR instead of ISR, which is via jasmonic acid. So, it would be possible to associate SA production by EPS384 and SAR activation but more studies should be performed to determine more precisely the nematode control mechanism involved.

P. fluorescens EPS895 synthesized siderophores and Plt. In previous studies (Badosa, 2001), genes related to PCA biosynthesis were detected in EPS895. Somehow, this strain did not synthesize PCA in LB broth, suggesting that production of this metabolite might be regulated by special biotic or environmental factors. Additionally, EPS895 did not reduce nematode egg hatching but produced a high juvenile mortality in LB broth. This suggests that Plt may not have a deleterious effect on eggs. The high juvenile mortality may be caused by anoxia or by another cause, such as production of a non-detected molecule with deleterious effect on juveniles, pH decrease, etc.

P. fluorescens CHA0 was used in experiments as a reference for positive *M. javanica* biocontrol effectiveness, and to compare the relative efficacy of strains EPS384 and EPS895 against *M. javanica*. CHA0 presented a wide range of synthesis of antibiotics and other molecules, since it synthesized siderophores, SA, IAA, Prn and HCN. Strain CHA0 was also able to solubilize inorganic phosphates and to hydrolize chitin, a major component of nematode egg wall. This metabolic activity may explain the reduction on *M. javanica* egg hatching and the high juvenile mortality produced by this strain *in vitro*.

M. javanica juvenile survival and egg hatching were not affected by presence of bacterial cells in the sterile sand test, therefore, bacterial cells did not have a direct deleterious effect on *M. javanica*, suggesting that these strains may reduce nematode infection in plants and *in vitro* either by production of a toxic metabolite or by a mechanism that indirectly affects the nematode.

Therefore, EPS384 may induce plant resistance (either SAR or ISR) and EPS895 may synthesize toxic metabolites that reduce *M. javanica* viability. CHA0 presents a broader range of toxic metabolites and other possible mechanisms, as it has been reported in literature. Siddiqui and Shaukat (2002) reported suppression of *M. javanica* by *Pseudomonas fluorescens* strain CHA0 via induction of plant resistance on tomato. In another study, Siddiqui *et al.* (2005) reported that the extracellular protease AprA of *P. fluorescens* CHA0 reduced both *M. incognita* egg hatching and juvenile survival *in vitro* and also suppressed root-knot development on tomato and soybean roots. Strain CHA0 is, therefore, a biocontrol agent that may have more than one mechanism of action to suppress *Meloidogyne* spp. on different plant hosts. It is also possible that EPS384 and EPS895 may have other mechanisms of action, and more research should be done to determine in more detail how these strains suppress *M. javanica* infection.

The application of a mixture of EPS384 and EPS895 on GF-677 inoculated with *M. javanica* did not increase biocontrol efficacy, which was similar to the efficacy observed when individual strains were applied. Although only two replicates of the experiment were performed, the application of a mixture of EPS384 and EPS895 seemed to reduce variability between experiments, and therefore increased consistency of biocontrol. However, more studies should be performed in order to confirm that the combination of strains leads to a reduction of variability. This is very important since low consistency is one of the major problems of biocontrol, and an obstacle to the applicability of biocontrol in field conditions (Meyer and Roberts, 2002). Ji *et al.* (2006) observed that combination of foliar applications of biocontrol agents with applications of PGPR strains in the roots improved suppression of bacterial diseases on tomato in some field trials, but consistency of biocontrol was too low since in some trials efficacy of combined treatments was similar or even lower than biocontrol strains applied alone.

P. fluorescens EPS384 and EPS895 not necessarily have a synergistic effect when applied in combination. Additionally, the population levels of EPS895 when it was applied in combination with EPS384 slightly

decreased compared to individual applications. This may be due to different rhizosphere competence of these two strains in the GF-677 rootstock rhizosphere, being EPS895 more susceptible to variations of rhizosphere conditions, and maybe this strain is less effective if its population levels in the rhizosphere are low. In contrast, EPS384 may have a higher root colonization ability than EPS895, and is therefore less susceptible to biotic and abiotic changes in the rhizosphere. When both strains were combined, it is likely that the effect of strain EPS895 was relatively less conspicuous, since its population was lower, and the additive effect of EPS384 and EPS895 was not sufficient to increase biocontrol efficacy.

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General Discussion and Conclusions

General Discussion

In this study, different *Pseudomonas fluorescens* strains were selected and characterized as biological control agents against two plant pathogens (*Phytophthora cactorum* and *Meloidogyne javanica*) in two different pathosystems (strawberry plants and the peach-almond hybrid GF-677 rootstock). These bacterial strains belonged to a collection of 58 *P. fluorescens* and *Pantoea agglomerans* strains that were selected in screening assays *in vitro*, *ex vivo* and/or *in planta*, characterized, and tested *in vitro* and/or *in planta* individually and in combination against the correspondant pathogen.

As it has been reported in literature, application of a single biological control agent (BCA) has obtained good results in biological control of bacterial (Han *et al.*, 2000; Stockwell *et al.*, 2002; Cabrefiga, 2004), fungal (Okamoto *et al.*, 2000; Heungens and Parke, 2001; Kurze *et al.*, 2001; Steddom *et al.*, 2002; Kamenski *et al.*, 2003) and nematode (Sharon *et al.*, 2001; Siddiqui and Ehteshamul-Haque, 2001; Siddiqui and Shaukat, 2003) plant pathogens. However, a general problem of biological control is its variability in efficacy and low consistency. Several authors have reported that some combinations of compatible biological control agents can improve biocontrol efficacy compared to single strain treatments (Raupach and Kloepper, 1998; Singh et al., 1999; Guetsky *et al.*, 2001; Guetsky *et al.*, 2002; Jetiyanon and Kloepper, 2002; Meyer and Roberts, 2002; Siddiqui and Shaukat, 2002b; Roberts *et al.*, 2005; Spadaro and Gullino, 2005; Jaizme-Vega *et al.*, 2006). For example, Guetsky *et al.* (2002) reported that combinations of compatible biological control agents increased biocontrol of *Botrytis cinerea* on both strawberry leaves and plants compared to single strain applications. These biocontrol agents acted

synergistically. Vestberg *et al.* (2004) observed some decrease of disease severity when strawberry plants inoculated with *Phytophthora cactorum* and *Phytophthora fragariae* were treated with compatible combinations of multiple beneficial microorganisms (*Pseudomonas fluorescens*, *Trichoderma harzianum*, *Bacillus subtilis*, *Glomus mosseae* and *Gliocladium catenulatum*). Overall, applications of mixtures of biological control agents, each with different mechanisms of action, is a way to improve biological control and reduce variability between experiments.

The present study shows two examples of biological control against two different plant pathogens, in which mixtures of bacterial strains are effective in disease control with an efficacy similar to applications of single strains. Application of mixtures of the selected strains produced different effects. *In vitro*, the inhibitory effect of strain combinations on *P. cactorum* germination was generally maintained or even reduced compared to the effect of individual strains, whereas in *P. cactorum*-inoculated strawberry plants and in GF-677 inoculated with *M. javanica*, the combination of strains maintained biocontrol efficacy and reduced variability.

Identification and selection of bacterial strains effective against *P. cactorum* in strawberry and against *M. javanica* in GF-677 rootstocks is the first step to obtain biological control strains that can be commercialized and used in commercial exploitations of these two plant species.

In Chapter I, an *ex vivo* screening method consisting of a detached leaf inoculation method was developed and applied to test a collection of bacterial strains against *P. cactorum* on strawberry detached leaves. The detached leaf inoculation method consisted of detached young strawberry leaves artificially inoculated with zoospore suspensions obtained from 18- to 25-day-old *P. cactorum* cultures that clearly developed infection necrosis 10 to 15 days after inoculation of the pathogen. This *ex vivo* method permitted to pre-select bacterial strains that were effective against *P. cactorum* infection and that were subsequently tested on strawberry plants, thus reducing the number of possible biocontrol strains to be tested in *in planta* assays. Since biocontrol of *P. cactorum* on detached leaves was consistent with biocontrol of the pathogen in strawberry plants, tedious and time-consuming screening assays in greenhouse using potted plants could be avoided, reducing the scale of whole plant screening assays to an optimal size. A similar procedure had been used in other pathosystems. Ellis *et al.* (1999) observed consistent inhibitory ability of *Pseudomonas fluorescens* 54/96 against the causal agent of damping-off *Pythium ultimum in situ* in pea seeds germination assays and *in vitro* in pathogen oospore germination and hyphal growth assays. Another example reported by Zhang *et al* (2002) is the consistency of

inhibition of *Peronospora tabacina* (the causal agent of blue mold of tobacco) by different PGPR strains *in vitro* in microtiter plate assays and *ex vivo* on detached leaves with reduction of blue mold in tobacco plants in greenhouse assays. Although the biocontrol ability of a bacterial strain in plants may be different to the inhibitory ability observed *in vitro* and *ex vivo* due to different factors that interact with the bacterial strain (environmental factors, edaphic factors, plant host, rhizosphere biotic and abiotic composition, pathogen species, available nutrients...), the main advantatge of *ex vivo* tests is that all these factors are controlled, and a more direct action of the bacterial strain upon the pathogen can be determined. The detached leaf inoculation method developed to select strains for biocontrol of *P. cactorum ex vivo* could also be used to test other bacterial strains against *P. cactorum* or other strawberry pathogens because it is a reliable method that can considerably reduce the experimental size. Similar *ex vivo* methods could be developed for other host plants and other pathosystems.

The effect of combinations of *P. fluorescens* strains EPS599, EPS817 and EPS894 on *P. cactorum* cyst germination reduction was similar to the effect of strains applied alone. In strawberry plants, mixtures of strains maintained the efficacy of disease suppression but improved consistency compared to strains applied individually. The results showed that EPS599 alone was more consistent in disease suppression than EPS817 and EPS894. It seems that EPS599 may be the principal strain responsible of the suppressive effect, and by combining this strain with another strain with a similar or lower efficacy the effect of both strains may lead to a reduction of variability. The triple combination, however, was not as effective as dual combinations containing EPS599. This may be explained by a possible antagonism among these three strains in the rhizosphere.

In Chapter II, selection of effective bacterial strains against *M. javanica* on the GF-677 rootstock was performed by means of a principal components analysis. The principal components analysis is an exploratory tool that permitted to summarize information of variables into three dimensions or principal components (PC) that provided a way to identify bacterial strains by their trend pattern. Therefore, the first principal component (PC1) was explained by *M. javanica* infection, the second principal component (PC2) by the *in vitro* antagonism of strains and the third principal component (PC3) by the metabolic activity of strains. The relationship between PC1 and PC3 was more interesting since it associated the synthesis of antibiotics and other molecules by strains with their effect on *M. javanica* infection. On the basis of these results, biocontrol of *M. javanica* by bacterial strains that possess a high

metabolic activity may result in a deleterious effect on nematode reproduction and viability, and subsequently, a reduction of nematode population, plant infection and gall formation by four possible ways: 1) a direct effect of toxic metabolites on larvae and juveniles that are developing inside the eggs would prevent egg hatching; 2) metabolites that are toxic to hatched second-stage juveniles may cause a high mortality rate of these juveniles, which in turn decreases root infection and gall formation; 3) metabolites that have a toxic effect on females would reduce the reproduction rate; and finally, 4) metabolites that would act indirectly by stimulation of plant defenses or by preventing the formation of feeding sites would also reduce gall formation and nematode reproduction. Principal components analysis distributed all 58 bacterial strains according to these three principal components, and allowed the selection of the best ones, which were furtherly studied. Selected *P. fluorescens* strains EPS384 and EPS895 and their combination were tested against the nematode in GF-677 rootstocks, but no increase in control efficacy by the combination of strains was observed. EPS384 presumably reduced *M. javanica* infection by induction of plant resistance, and EPS895 by antibiosis, but no synergism was observed when both strains were combined, although variability was reduced. Since EPS384 population on roots was higher than population of EPS895 when both strains were applied together, it may be possible that EPS384 has a better root colonization ability than EPS895, and in some situations the presence of the former would reduce the population of the latter, which would be more susceptible to variable rhizosphere conditions. This competition for colonization sites may result in fluctuations of the population levels of these strains in the rhizosphere, but since biocontrol efficacy is maintained and variability decreases when both strains are combined compared to strains applied alone, it is likely that both strains occupy different niches in the GF-677 rhizophere.

As it has been stated above, combinations of compatible strains with different mechanisms of action can improve consistency of biocontrol. Taking into account the results obtained in this study in the two different pathosystems, some combinations of strains result in a reduction of variability, and hence, in a higher consistency, although biocontrol efficacy may remain the same as that reached by application of individual strains. In general, some combinations of strains can be equally or even less effective than individual applications of these strains. This is probably caused by a reduced effectiveness of the mechanism of action of one or more strains of the mixture due to their lower adaptability to the rhizosphere, and leads to a reduced or a lack of additive effect of both strains when applied together. Additionally, as

the results observed in Chapter II indicate, the population of one strain of the mixture in the rhizosphere decreases while the other strain appears unaffected. Nevertheless, a more rigorous study with multiple replicates should be performed to confirm this putative reduction of variability and subsequent increase in control consistency. Other effective strains and their combinations should also be tested against *P. cactorum* and *M. javanica* in future studies, as well as combinations of these new strains with the strains selected in this study. Moreover, the strains selected in this study and their combinations should be tested against these pathogens or other plant pathogens in different host plants. It would also be interesting to study the mechanism of action of these strains and their colonization ability in more detail. For instance, the detection *in situ* of production of antibiotics, enzymes or other molecules that could have a role in biocontrol, or the mechanism of induction of plant resistance by biocontrol strains.

The development of *ex vivo* methods with applicability in plant diseases caused by nematodes is more difficult, and selection of biocontrol strains is performed using whole plants. However, selection of biocontrol strains using seedlings and gnotobiotic systems under *in vitro* conditions may be an alternative to *ex vivo* methods that would permit to reduce the scale of screening assays. The use of seedlings has been developed mostly in shrubs (McBride *et al.*, 1999) and herbaceous plants (Aalten *et al.*, 1998) for determination of host resistance to *Meloidogyne* spp. and other nematode species, although *in vitro* experiments performed on micropropagated woody plants have also been reported (Huettel and Hammerschlag, 1993; Van Vuuren and Woodward, 2001). A gnotobiotic system similar to that described on tomato by Simons *et al.* (1996) could be used for screening biocontrol agents against *Meloidogyne* spp. This *in vitro* system may also be used with micropropagated woody plants. Nevertheless, the development of a method to select biocontrol strains against plant-pathogenic nematodes on micropropagated woody plants or on seedlings may be rather costful and it is likely that its reliability may be low. Therefore, the best way to select biocontrol strains against plant-pathogenic nematodes on woody plants are whole plant assays under greenhouse conditions using larger containers with young plants.

After selecting effective strains against a pathogen, dose-response relationship studies should be performed to determine the optimal dose of biocontrol agent to effectively control the pathogen, following the development of mass production, formulation and monitoring methods to obtain a preparation of these biocontrol agents that would be easily applied in the field and would improve survival of biocontrol strains in the soil and also allow their specific

detection and quantification once applied (Montesinos, 2003). The use of combinations of compatible bacterial strains is a promising alternative to the application of individual strains, and may be the best way to improve biological control. The integration of mixtures of biocontrol agents with other control methods would allow a more effective and consistent control of a broader range of target plant pathogens. On the other hand, there are some production and quality control difficulties that are increased when a mixture of more than one biocontrol agent is involved. It would be necessary to assess specific formulations for multiple biocontrol agents, and the shelf life of each organism has to be ensured. Additionally, development and production costs would be increased, and registration is likely to be more difficult and expensive, as each biocontrol agent must be registered independently. Therefore, the acceptance of a biocontrol combination would depend on its efficacy and the resulting economic benefit that justify the use of combinations over application of individual biocontrol agents (Meyer and Roberts, 2002). Currently, this may be a problem to the development of more biocontrol products based on mixtures of biocontrol agents in countries with a strict registration procedure for biocontrol agents, e. g. the European Union.

Finally, practical considerations are very important. In the case of fruit tree rootstocks the challenge is even greater since plants are established in the field for many seasons or decades. The control strategy and the use of bacterial combinations should be oriented to protect the plant following establishment in the field during its first or second year when the plant is most vulnerable to nematode attack. It is likely that repeated applications will be necessary and costs will limit continued use. The selection of adequate strains that will survive in the rhizosphere, which is a fragile system, and will be effective during this establishment period is a minimum requirement. Biocontrol strains, applied individually or in combination, will be subject to changes in soil temperature, irrigation, flooding effects of rains, fertilization with inorganic or organic amendments (e.g. manure), weed control practices, as well as many other agronomic practices, all of which can affect the composition of rhizosphere microbiota including beneficial biocontrol agents. Field trials at a greater scale (mesoscale) would confirm the effectiveness of these biocontrol agents (individually and combined) against *P. cactorum*, *M. javanica* and/or other plant pathogens. Combinations of these biocontrol agents with other biocontrol agents and/or chemical pesticides should also be tested in field trials.

Conclusions

1. *Phytophthora cactorum* is able to infect and colonize detached strawberry leaves and develop infection symptoms in a short period of time (10 days after inoculation). Inoculation of wounded young strawberry leaves from susceptible cultivars with zoospore suspensions from 18- to 25-day-old cultures of a virulent *P. cactorum* strain are the optimal conditions for infection in *ex vivo* assays.

2. The detached leaf inoculation method has proved to be useful for screening bacterial strains as biological control agents of Phytophthora root rot. Effectiveness of bacterial strains in infection reduction in *ex vivo* assays is consistent with *in planta* disease control.

3. *Pseudomonas fluorescens* EPS599, EPS817, EPS894, Ps15 and SBW25, selected from a collection of 58 *P. fluorescens* and *Pantoea agglomerans* strains, reduce *P. cactorum* infection on detached strawberry leaves and disease severity in strawberry plants, presenting different *in vitro* antagonistic activity against the pathogen. Strains EPS599, EPS817 and EPS894 are proposed as potential biological control agents of *P. cactorum* in strawberry. Combinations of these strains maintain efficacy and reduce variability of *P. cactorum* biocontrol.

4. *P. fluorescens* EPS599 produces pyoluteorin, *P. fluorescens* EPS894 produces pyoluteorin and phenazine-1-carboxylic acid, and *P. fluorescens* EPS817 produces hydrogen cyanide and 2,4-diacetylphloroglucinol. All strains can also synthesize siderophores.

5. Bacterial cells and cell-free culture extracts of *P. fluorescens* EPS599, EPS817 and EPS894 reduce *P. cactorum* cyst germination *in vitro*. The addition of nutrients to cell-free culture extracts partially restores germination, suggesting that antibiosis and nutrient depletion are responsible of this reduction. Strains EPS599 and EPS894 also reduce *P. cactorum* mycelial growth *in vitro*.

6. *P. fluorescens* EPS384, EPS895 and CHA0, selected from a collection of 58 *P. fluorescens* and *P. agglomerans* strains reduce *M. javanica* infection on GF-677 rootstocks, although efficacy is variable depending on the experiment. These strains also reduce *M. javanica* egg hatching (EPS384 and CHA0) and juvenile survival *in vitro*.

7. *P. fluorescens* EPS384 produces salicylic acid and presents inorganic phosphate solubilization ability, *P. fluorescens* EPS895 produces pyoluteorin, and *P. fluorescens* CHA0 produces salicylic acid, pyoluteorin, 1-indole-3-acetic acid, pyrrolnitrin, 2,4-diacetylphloroglucinol, hydrogen cyanide, and is able to hydrolyze chitin and solubilize inorganic phosphate. These strains also synthesize siderophores.

8. *P. fluorescens* EPS384, EPS895 and their combination reduce *M. javanica* infection on GF-677. The combination of strains does not increase biocontrol efficacy, but it reduces variability between experiments. *P. fluorescens* EPS384 induces systemic resistance on GF-677 and reduces gall formation in *M. javanica*-inoculate plants. This suggests that a probable mechanism of action of strain EPS384 is induction of plant resistance, maybe via salicylic acid.

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Annexes

Annex I. Media and buffers

Modified V8 agar (Dhingra and Sinclair, 1987)

Tomato concentrate	12.5 g
$CaCO_3$	2.85 g
Agar	16 g
Distilled water	1000 ml

Adjust to pH 6.5-7.0
Sterilization by autoclaving at 121ºC for 20 min

Luria-Bertani (LB) broth and agar (Maniatis *et al*., 1982)

Triptone	10 g
NaCl	10 g
Yeast extract	5 g
Agar (for LB agar)	15 g
Distilled water	1000 ml

Sterilization by autoclaving at 121ºC for 20 min

Potato-dextrose broth (PDB) and agar (PDA)

Potato infusion	200 g
D(+)Glucose	20 g
Agar (for PDA)	15 g
Distilled water	1000 ml

Adjust to pH 5.6
Sterilization by autoclaving at 121ºC for 20 min

Müeller-Hinton (MH) agar

Meat extract	2 g
Hidrolyzed casein	17.5 g
Starch	1.5 g
Agar	15 g
Distilled water	1000 ml

Sterilization by autoclaving at 121ºC for 20 min

Tryptone-soy agar (TSA) amended with AZCL (Nielsen *et al.*, 1998)

Tryptone soy agar (Oxoid Ltd)	40 g
Azurine-dyed cross-linked chromogenic substrate	1 g
Distilled water	1000 ml

Sterilization by autoclaving at 121ºC for 20 min

Pikovskaya agar (Nautiyal, 1999)

Glucose	10 g
$Ca_3(PO_4)_2$	5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
NaCl	0.2 g
KCl	0.2 g
$(NH_4)_2SO_4$	0.5 g
$MnSO_4 \cdot 1H_2O$	2 mg
$FeSO_4 \cdot 7H_2O$	2 mg
Yeast extract	0.5 g
Agar	15 g
Distilled water	1000 ml

Adjust to pH 7.0
Sterilization by autoclaving at 121ºC for 20 min

NBRIY (Nautiyal, 1999)

Glucose	10 g
$Ca_3(PO_4)_2$	5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
NaCl	0.2 g
KCl	0.2 g
$(NH_4)_2SO_4$	0.5 g
$MnSO_4 \cdot 1H_2O$	2 mg
$FeSO_4 \cdot 7H_2O$	2 mg
Agar	15 g
Distilled water	1000 ml

Adjust to pH 7.0
Sterilization by autoclaving at 121ºC for 20 min

NBRIP agar (Nautiyal, 1999)

Glucose	10 g
$Ca_3(PO_4)_2$	5 g
$MgSO_4 \cdot 7H_2O$	0.25 g
$MgCl_2 \cdot 6H_2O$	5 g
KCl	0.2 g
$(NH_4)_2SO_4$	0.1 g
Agar	15 g
Distilled water	1000 ml

Adjust to pH 7.0
Sterilization by autoclaving at 121ºC for 20 min

Chitin medium (Frändberg and Schnürer, 1998; Rodríguez-Kábana *et al.*, 1983)

K_2HPO_4	2.7 g
KH_2PO_4	0.3 g
$MgSO_4 \cdot 7H_2O$	0.7 g
NaCl	0.5 g
KCl	0.5 g
$CaCl_2 \cdot 1H_2O$	0.13 g
Yeast extract	3 g
Colloidal chitin	1.5 g
Agar	20 g
Distilled water	1000 ml

Sterilization by autoclaving at 121ºC for 20 min

Glucose minimum medium (GMM)

Glucose	5 g
NH_4Cl	1 g
KH_2PO_4	3 g
Na_2HPO_4	2.4 g
NaCl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Distilled water	1000 ml

Adjust to pH 6.5
Sterilization by filtration with a 0.22 μm porus membrane

Tris-Acetate-EDTA (TAE) buffer 50%

Tris base	121 g
Glacial acetic acid	28.55 ml
EDTA 0.5 M pH 8.0 (sterile)	50 ml

Dissolve in 350 ml of milliQ water. Adjust to pH 7.5-7.8 with HCl. Bring up to 500 ml with milliQ water.

Annex II. Tables

Table I. Characteristics of 58 *Pseudomonas fluorescens* and *Pantoea agglomerans* strains used in Chapter I to evaluate potential biocontrol strains against *Phytophthora cactorum* in strawberry.

		Inorganic phosphate solubilization[x]			*In vitro* antagonism[y]		*Ex vivo* infection severity[z]	
Bacterial strain	Chitinolytic activity	Pikovskaya	NBRIY	NBRIP	PDA	MH	Camarosa	Diamante
EPS227	-	-	-	+	2.7	3.2	27.8	48.1
EPS231	+	-	-	-	5.7	8.8	42.6	72.2
EPS263	+	+	+	+	13.0	13.3	33.3	61.1
EPS282	-	+	+	+	3.4	3.5	20.4	29.6
EPS290	+	-	-	+	2.7	nd	18.5	24.1
EPS291	+	-	-	-	1.8	nd	63.0	50.0
EPS302	+	-	+	+	3.2	5.4	24.1	83.3
EPS317	-	-	-	-	3.6	4.1	0.0	22.2
EPS326	-	+	+	+	2.6	6.2	25.9	40.7
EPS328	-	+	+	+	4.0	9.2	1.9	55.6
EPS340	+	+	+	-	4.1	7.4	13.0	44.4
EPS341	+	-	+	+	5.1	6.1	27.8	61.1
EPS353	-	+	+	+	0.0	7.6	46.3	61.1
EPS384	-	+	+	+	0.2	7.1	53.7	63.0
EPS394	-	+	+	+	0.0	0.9	79.6	59.3
EPS424	+	+	+	+	3.6	9.0	37.0	59.3
EPS427	-	+	+	+	0.0	1.4	70.4	31.5
EPS435	-	+	+	+	0.0	0.9	48.1	59.3
EPS453	-	+	+	+	0.0	1.0	29.6	37.0
EPS454	-	+	+	+	0.0	6.1	5.6	13.0
EPS458	-	+	+	+	2.4	0.0	1.9	13.0
EPS475	-	+	+	-	0.0	4.8	24.1	13.0
EPS495	-	+	+	+	0.0	2.0	44.4	14.8
EPS512	-	+	+	+	0.0	3.4	42.6	24.1
EPS514	-	+	+	+	0.0	2.2	40.7	24.1
EPS519	-	+	+	+	0.0	3.8	51.9	37.0
EPS532	-	+	+	+	4.1	8.0	90.7	35.2
EPS537	+	+	+	+	3.4	8.6	61.1	46.3
EPS539	-	+	+	+	2.7	9.1	13.0	20.4
EPS547	-	+	+	+	0.8	5.0	24.1	55.6
EPS550	+	+	+	-	6.7	6.9	22.2	3.7
EPS560	-	-	-	-	1.3	0.0	25.9	27.8
EPS595	-	-	-	-	0.0	2.6	5.6	24.1
EPS599	+	-	+	+	0.0	10.5	9.3	18.5
EPS607	-	-	+	+	0.0	3.0	25.9	40.7
EPS615	-	+	+	+	0.0	4.5	11.1	50.0
EPS622	-	+	+	+	0.0	0.9	77.8	38.9
EPS807	-	-	-	-	6.1	9.4	66.7	35.2
EPS808	-	-	-	-	4.8	8.5	94.4	51.9
EPS809	-	-	-	-	5.4	9.3	74.1	57.4
EPS812	-	-	-	-	5.4	6.1	3.7	27.8
EPS817	-	-	-	-	5.8	6.1	0.0	33.0
EPS818	-	-	-	-	5.8	6.1	5.6	48.1
EPS894	-	-	-	-	11.1	6.6	1.9	16.7
EPS895	-	-	-	-	13.6	6.6	5.6	35.2
EPS944	-	-	-	-	11.5	6.9	14.8	27.8

Table I. Continuation.

Bacterial strain	Chitinolytic activity	Inorganic phosphate solubilization[x]			*In vitro* antagonism[y]		*Ex vivo* infection severity[z]	
		Pikovskaya	NBRIY	NBRIP	PDA	MH	Camarosa	Diamante
CHA0	+	+	+	+	2.9	1.7	16.7	57.4
JBR170	+	-	-	-	7.3	5.1	33.3	63.0
WB52	-	+	+	+	0.3	0.2	15.7	53.7
Ps15	+	+	+	+	2.6	1.6	18.5	29.6
Ps31	+	-	-	-	5.8	3.6	55.6	25.9
JMP1284	+	+	+	+	5.5	3.2	3.7	66.7
M480R	+	-	-	+	0.0	8.9	22.2	13.0
SBW25	-	+	+	+	0.0	9.1	0.0	16.7
BL915	+	-	-	-	2.9	10.1	13.0	31.5
Qc69-80	-	+	-	+	1.8	6.3	5.6	20.4
Q2-87	-	+	+	+	6.9	6.3	7.4	13.0
Q4-87	-	+	+	+	6.8	6.5	7.4	29.6

[x] Inorganic phosphate solubilization ability on three different media.

[y] *In vitro* antagonistic activity against *P. cactorum* on PDA and MH media. Values correspond to the mean inhibition zone (mm) of 12 replicates for each bacterial strain and medium. Data used in the second part of Chapter I correspond to the mean of the *in vitro* antagonistic activity of strains in the two media tested.

[z] *Ex vivo* infection severity (%) on detached strawberry leaves of cvs. Camarosa and Diamante treated with bacterial strains and inoculated with *P. cactorum*. Values correspond to the mean of three sets of six leaflets for each bacterial strain per cultivar.

Symbols correspond to: (+) positive activity; (-) negative activity; nd, not determined.

Table II. Characteristics of 58 *Pseudomonas fluorescens* and *Pantoea agglomerans* strains used in Chapter II to evaluate potential biocontrol strains against *Meloidogyne javanica* in GF-677 rootstocks by means of principal components analysis.

	Variables of the principal components analysis				Principal components		
Bacterial strain	Galls[w]	Reproduction index[x]	Metabolic activity[y]	*In vitro* antagonism[z]	PC1	PC2	PC3
EPS227	109.2	14.5	3	9	0.200	0.424	-0.823
EPS231	45.8	32.9	3	8	1.285	-0.894	-0.575
EPS263	54.8	11.4	8	10	-1.030	1.575	1.668
EPS282	109.0	21.0	5	3	0.084	-0.486	0.749
EPS290	91.8	18.8	4	4	0.044	-0.417	0.067
EPS291	97.6	21.1	3	4	0.407	-0.723	-0.425
EPS302	-18.2	12.2	5	1	-1.280	-0.470	0.440
EPS317	223.0	34.0	2	17	2.792	0.477	-1.267
EPS326	-94.6	8.8	5	6	-1.641	0.354	-0.092
EPS328	-50.8	22.6	5	8	-0.271	-0.083	0.124
EPS340	-40.6	21.3	4	3	-0.419	-0.918	-0.116
EPS341	165.4	21.0	4	4	0.551	-0.431	0.265
EPS353	-6.8	20.1	5	3	-0.515	-0.614	0.482
EPS384	-170.8	5.0	5	13	-1.931	1.500	-0.754
EPS394	-110.8	17.2	4	0	-1.208	-1.235	-0.157
EPS424	-114.2	19.6	5	4	-0.995	-0.603	0.178
EPS427	-43.0	19.6	5	0	-0.871	-1.088	0.578
EPS435	101.0	31.8	5	0	0.733	-1.574	1.106
EPS453	114.4	28.6	5	4	0.748	-0.771	0.834
EPS454	299.2	34.5	5	0	1.848	-1.423	1.584
EPS458	-81.2	25.5	5	1	-0.538	-1.342	0.538
EPS475	-48.0	26.2	4	0	-0.224	-1.661	0.139
EPS495	-81.5	12.3	6	3	-1.619	-0.095	0.727
EPS512	-79.5	30.4	4	2	0.058	-1.656	0.022
EPS514	65.3	10.7	5	1	-1.014	-0.253	0.595
EPS519	160.8	26.6	5	1	0.654	-1.029	1.083
EPS532	38.6	9.2	5	4	-1.101	0.240	0.327
EPS537	270.2	21.7	6	5	0.818	0.203	1.534
EPS539	118.4	11.2	5	8	-0.380	0.844	0.290
EPS547	141.8	20.0	5	2	0.106	-0.526	0.864
EPS550	260.8	6.4	4	13	0.310	1.908	-0.336
EPS560	-71.8	18.3	1	7	-0.117	-0.739	-2.117
EPS595	-62.4	11.4	1	1	-0.911	-1.217	-1.850
EPS599	55.2	26.3	4	3	0.407	-1.059	0.181
EPS607	-96.0	10.1	4	4	-1.489	-0.203	-0.495
EPS615	-130.4	25.2	4	4	-0.477	-1.135	-0.303
EPS622	4.2	9.7	5	0	-1.422	-0.439	0.506
EPS807	1.2	18.4	3	15	0.311	0.923	-1.356
EPS808	286.6	21.1	3	15	1.825	1.210	-0.689
EPS809	209.6	26.8	3	18	2.065	1.205	-0.939
EPS812	154.2	14.7	3	17	0.825	1.674	-1.212
EPS817	-0.6	27.1	3	15	0.976	0.414	-1.207
EPS818	-132.2	12.7	3	17	-0.640	1.345	-1.869
EPS894	-127.0	30.0	3	7	0.220	-1.143	-0.940
EPS895	-127.7	5.8	2	8	-1.449	0.231	-1.975
EPS944	211.0	17.3	2	9	1.041	0.238	-1.097

Table II. Continuation.

Bacterial strain	Variables of the principal components analysis				Principal components		
	Galls[w]	Reproduction index[x]	Metabolic activity[y]	*In vitro* antagonism[z]	PC1	PC2	PC3
CHA0	-106.0	6.2	11	15	-2.391	2.916	2.556
JBR170	92.8	13.2	4	12	0.017	1.102	-0.520
WB52	18.0	12.6	5	8	-0.731	0.606	0.097
Ps15	-5.8	21.8	6	11	-0.134	0.662	0.569
Ps31	132.2	13.3	4	10	0.105	0.860	-0.310
JMP1284	41.3	15.7	7	13	-0.449	1.570	0.986
M480R	-173.2	27.0	3	6	-0.274	-1.190	-1.032
SBW25	230.2	19.8	4	6	0.855	0.038	0.262
BL915	292.4	25.4	4	16	2.077	1.299	-0.117
Qc69-80	101.2	13.4	4	6	-0.231	0.210	-0.131
Q2-87	590.6	40.4	6	10	3.983	0.359	2.254
Q4-87	225.4	16.5	6	9	0.412	1.032	1.100

[w] Root galling correspond to the difference between the number of galls per root system of each treatment and the non-treated control.
[x] Reproduction index (RI) correspond to the mean number of eggs per root system of each treatment divided by the number of inoculated eggs.
[y] Metabolic activity correspond to the summatory of qualitative metabolic characteristics, being production (1) and no production (0), and comprised the ability to synthesize secondary metabolites and other molecules (HCN, IAA, siderophores, DAPG, PCA, SA, Plt and Prn) and the ability to hydrolize different polymers (chitin, cellulose, β-1,3-glucan, mannan and xylan) and inorganic phosphates.
[z] *In vitro* antagonism correspond to the summatory of qualitative results, accounting for positive *in vitro* antagonism (1) or negative *in vitro* antagonism (0), observed when bacterial strains were tested for *in vitro* antagonistic activity against different plant pathogens on agar culture plates in different media. Pathogens included *Phytophthora cactorum* (see Table I, Annex II), *Erwinia amylovora*, *Stemphylium vesicarium*, *Pseudomonas syringae* and *Penicillium expansum* (Montesinos *et al*., 1996; Bonaterra, 1997; Francés, 2000; Badosa, 2001; Bonaterra *et al*., 2003; Cabrefiga, 2004).

Table III. Eigenvectors of the first three principal components obtained in the principal components analysis to evaluate potential *Pseudomonas fluorescens* and *Pantoea agglomerans* biocontrol strains against *Meloidogyne javanica* in GF-677 (Chapter II).

Variables	Eigenvectors		
	PC1	PC2	PC3
Gall formation (Galls per root system)	0.6814	0.2317	0.3238
Reproduction index (eggs per root/inoculum)	0.6310	-0.4740	0.1437
Metabolic activity	-0.2544	0.2912	0.8757
In vitro antagonism	0.2698	0.7980	-0.3282