Universitat de Girona

FIRE BLIGHT (ERWINIA AMYLOVORA) OF ROSACEOUS PLANTS. PATHOGEN VIRULENCE AND SELECTION AND CHARACTERIZATION OF BIOLOGICAL CONTROL AGENTS

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TESI DOCTORAL

FIRE BLIGHT (*Erwinia amylovora*) OF ROSACEOUS PLANTS. PATHOGEN VIRULENCE AND SELECTION AND CHARACTERIZATION OF BIOLOGICAL CONTROL AGENTS.

Memòria presentada per optar al Grau de Doctor per la Universitat de Girona, per

Jordi Cabrefiga i Olamendi

2004

Emili Montesinos Seguí i **Anna Bonaterra Carreras**, Catedràtic i Professor Titular de l'Àrea de Producció Vegetal, membres del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària i del Institut de Tecnologia Agroalimentària de la Universitat de Girona,

CERTIFIQUEN:

Que el llicenciat en Biologia Jordi Cabrefiga Olamendi ha dut a terme, sota la seva direcció, el treball amb el títol "Fire blight (*Erwinia amylovora*) of rosaceous plants. Pathogen virulence and selection and characterization of biological control agents", 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 14 d'abril de 2004.

Vist-i-plau directors de la Tesi

Dr. Emili Montesinos Seguí

Dra. Anna Bonaterra Carreras

Emili Montesinos Seguí, Catedràtic de Producció Vegetal de la Universitat de Girona, i director dels projectes de recerca 'Estudios epidemiológicos y evaluación de estrategias de control integrado de fuego bacteriano (*Erwinia amylovora*) en España' (Ref. AGF98-0402-C03-01) i 'Optimización de los métodos de detección, prevención y control del fuego bacteriano de las rosáceas en España' (Ref. AGL2001-2349-C03-01) de la Comisión Interministerial de Ciencia y Tecnologia, en els que es circumscriu la tesi doctoral titulada 'Fire blight (*Erwinia amylovora*) of rosaceous plants. Pathogen virulence and selection and characterization of biological control agents' amb el número de registre 307 de 5 de maig de 2000 realitzada per **Jordi Cabrefiga Olamendi**,

DECLARA QUE: Aquesta tesi està sotmesa a la propietat intel·lectual compartida amb els investigadors del grup de Patologia Vegetal i del Institut de Tecnologia Agroalimentària de la Universitat de Girona que participen en els esmentats projectes (Article 2. apartat 2, RD 1326/2003 de 24-10-2003; Llei de la Propietat Intel·lectual, RD 1/1996 de 12-04-1996).

I per a què consti als efectes oportuns, signen la present a Girona, el 14 d'abril de 2004.

Vist-i-plau

Dr. Emili Montesinos Seguí

Jordi Cabrefiga Olamendi

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RESUM

El foc bacterià, causat pel bacteri *E. amylovora*, és una malaltia molt important a nivell comercial i econòmic perquè afecta a les plantes de la família de les rosàcies i és especialment agressiva en pomera (*Pyrus malus*), perera (*Pyrus communis*) i plantes ornamentals (*Crataegus*, *Cotoneaster* o *Pyracantha*). Aquesta malaltia està distribuïda per tot el món en zones climàtiques temperades d'Amèrica del Nord, Nova Zelanda, Japó, Israel, Turquia i Europa. A Espanya, el foc bacterià va ser detectat per primera vegada el 1995 al nord del País (Euzkadi) i més tard, en nous focus apareguts en altres zones.

L'objectiu principal d'aquesta tesi doctoral va ser la selecció d'agents de biocontrol del foc bacterià i per aquesta raó es van seleccionar un grup reduït de soques d'*E. amylovora* i es van posar a punt diversos patosistemes *ex vivo* i *in planta* per ser utilitzats en el procés de selecció d'agents de biocontrol del foc bacterià. També es va estudiar l'existència de trets o patrons específics dels antagonistes associats a la inhibició de la infecció causada per *E. amylovora* i els possibles mecanismes d'acció involucrats en el biocontrol d'*E. amylovora* per una de les millors soques.

En el present treball, es van caracteritzar 53 soques d'*E. amylovora* de diferents orígens (localització geogràfica i planta hoste) en relació al tipus de colònia en diferents medis de cultiu, detecció per PCR i ELISA, perfils metabòlics utilitzant els sistemes API i Biolog GN, patogenicitat en fruits immadurs de pera i diversitat genètica mitjançant RFLPs del gen *ams* i de l'ADN genòmic. Les soques d'*E. amylovora* es van mostrar molt homogènies per a totes aquestes característiques. Tot i això, es van observar diferències principalment en els perfils metabòlics, MRFLPs de l'ADN genòmic i patogenicitat en peres immadures. La variació en els MRFLPs de l'ADN genòmic es va relacionar amb la distribució geogràfica, mentre que la variació en els perfils d'utilització de fonts de carboni semblaven estar relacionats amb la planta hoste per la tendència a agrupar les soques en dos grups, un composat principalment per soques aïllades de fruiters i l'altre per soques aïllades de plantes ornamentals. Es va observar major diversitat entre soques a nivell de virulència en peres immadures. Estudis basats en la relació dosi-malaltia i en corbes de progressió de la malaltia van permetre la classificació de les soques en funció de la seva agressivitat (dosi mitjana efectiva, incidència màxima de la malaltia, taxa de progressió de la malaltia), infectivitat (dosi infectiva mínima, moment d'inici de la infecció) o de la seva virulència en termes generals (índex de virulència compost, àrea sota la corba de progressió de la malaltia). Les soques que van presentar la major virulència van ser EPS101, UPN513, UPN544, USV1043, USV4512 i USV4576.

D'acord amb aquests resultats, les soques CUCM273 i EPS101 d'*E. amylovora* van ser elegides per realitzar la selecció d'agents de biocontrol. La soca EPS101 va ser seleccionada per haver estat aïllada de la nostra zona d'influència, a Lleida, i perquè presentava les característiques comunes de la majoria de soques d'*E. amylovora*. Per contra, la soca

CUCM273 va ser aïllada als Estats Units i es va seleccionar per ser probablement una de les soques més divergents, ja que, difereix de la resta en el serotip i en el tipus de colònia (blanca, convexe, brillant i mucosa).

El segon objectiu va ser l'optimització dels patosistemes que s'utilitzarien posteriorment en la selecció d'agents de biocontrol del foc bacterià i en estudis d'interacció. Aquests patosistemes consistien en assaigs d'infecció en fruits immadurs, flors i brots. Es van provar diferents mètodes d'inoculació i concentracions del patogen per cada assaig. Els mètodes optimitzats van consistir en la inoculació del patogen mitjançant la deposició d'una suspensió calibrada en les ferides produïdes en els fruits immadurs i en les fulles, en l'assaig en brots, o en la superfície del hipàntium de les flors en l'assaig en flors.

El tercer objectiu va ser la selecció d'agents de biocontrol. Per aquest motiu, es van provar al voltant de 533 aïllats, principalment de les espècies *Pseudomonas fluorescens* i *Erwinia herbicola* (sinònim de *Pantoea agglomerans*), obtinguts de flors, fruits i fulles de plantes rosàcies, utilitzant l'assaig en fruits immadurs de pera. Deu aïllats seleccionats en aquest assaig van ser posteriorment avaluats en el control de les infeccions causades per *E. amylovora* en flors i brots en condicions ambientals controlades. La soca EPS62e va reduir consistentment les infeccions en flors i brots presentant uns nivells de control equivalents als obtinguts mitjançant el tractament amb productes bactericides químics.

Amb la finalitat de determinar si existien trets o patrons característics en els antagonistes, associats a la inhibició de les infeccions causades per *E. amylovora* en peres immadures, es van caracteritzar les soques més eficaces utilitzant el sistema API, antagonisme *in vitro* contra diferents patògens de plantes, producció de compostos interessants (antibiòtics, sideròfors, quitinases, àcid indolacètic), nivell d''inhibició de les infeccions en fruits immadurs de pera, reacció hipersensible i activitat nucleadora de gel. Es van estudiar les associacions entre les característiques de les soques i la seva capacitat de biocontrol mitjançant l'anàlisi de correspondències. No es van observar relacions entre l'eficàcia en la inhibició de les infeccions causades per *E. amylovora* en fruits immadurs de pera i les característiques de les soques.

La soca EPS62e es va identificar com *P. fluorescens*, no va produir cap antibiòtic conegut ni cianhídric, àcid inolacètic, o quitinases. Tampoc va presentar activitat nucleadora de gel ni va induir reacció hipersensible en tabac. La soca va ser tolerant a fungicides i insecticides, lleugerament tolerant a kasugamicina i estreptomicina, encara que va ser sensible a fosetil d'alumini i a biocides d'ampli espectre com sulfat de coure o oxiclorur de coure. Aquesta soca va presentar un limitat espectre d'activitat inhibidora *in vitro* contra patògens bacterians de plantes i contra la major part de soques de *E. amylovora*, que es va atribuir predominantment a la producció de sideròfors. Tanmateix va presentar un ampli espectre d'activitat en assaigs *ex vivo* d'inhibició d'infeccions per soques d' *E. amylovora* en fruits immadurs. La soca EPS62e va presentar un patró electroforètic característic de fragments de macrorestricció de l'ADN genòmic amb l'enzim *Swa* I. Aquest patró va permetre diferenciar-la d'altres aïllats, soques tipus i agents de biocontrol de la mateixa espècie.

També es va estudiar la influencia de diferents concentracions de la soca EPS62e i del patogen sobre el control del foc bacterià. La relació entre la dosi efectiva mitjana de l'agent de biocontrol respecte a la d'*E. amylovora* (K_z/K_x) es va utilitzar per comparar l'eficiència de la soca EPS62e entre diferents varietats de perera i òrgans de la planta. En funció d'aquest paràmetre, la major eficiència en la inhibició d'infeccions d' *E. amylovora* es va obtenir en fruits immadurs de la varietat Conference on una cèl·lula de la soca EPS62e va ser capaç d'inactivar vint cèl·lules d' *E. amylovora*. La soca EPS62e també va ser altament eficaç en peres immadures de les varietats Passe Crassane i Blanquilla amb relacions d'1 i de 40, respectivament. Per contra, va ser poc eficaç en fruits i flors de la varietat Doyenne du Comice, amb una relació de 10^3, i en flors de la varietat Conference, amb valors de 570.

L'últim objectiu del present estudi va ser la determinació dels mecanismes d'acció involucrats en el biocontrol d' *E. amylovora* mitjançant EPS62e. L'antibiosi va ser descartada perquè la soca no va produir cap dels antibiòtics descrits en *P. fluorescens* ni va inhibir a la majoria dels bacteris fitopatógens ni soques d'*E. amylovora* en assaigs de difusió *in vitro* en diferents medis de cultiu. Contràriament, es va observar que era necessària la interacció directa en el procés d'inhibició mitjançant el contacte cèl·lula-cèl·lula. Els estudis d'interacció realitzats a diferents concentracions de ambdós bacteris i a diferents relacions e de la soca EPS62e i EPS101 van permetre estimar la taxa de creixement relatiu d' *E. amylovora* en funció del increment de la relació EPS62e:*E. amylovora*. Es va observar que aquest paràmetre era útil per comparar l'eficàcia de les soques antagonistes.

Es van obtenir evidències de la implicació de la competència per nutrients en observar una lleu inhibició del creixement d' *E. amylovora* quan la soca EPS62e i *E. amylovora* estaven físicament separades per una membrana semipermeable, però només en suc de pera diluït o quan la relació EPS62e:*E. amylovora* era molt elevada. A més, la soca EPS62e va presentar una bona habilitat en la competència per nutrients perquè va presentar una major velocitat màxima de creixement (μ_{max}) i afinitat per nutrients (menor K_s) que *E. amylovora* en suc de fruits immadurs de pera, així com un alt índex de solapament de nínxol (*NOI*), demostrant que es capaç d'utilitzar la major part de les fonts de carboni utilitzades per *E. amylovora*.

La implicació de la inducció de resposta defensiva en la planta es va avaluar com a possible mecanisme en la inhibició d'*E. amylovora* mitjançant la soca EPS62e. En aquest cas, la soca va mostrar la capacitat de retardar les infeccions causades per *E. amylovora* en plantes on prèviament s'havia infiltrat la soca EPS62e en el mesòfil de fulles. Tanmateix, es va observar un efecte inconsistent dels resultats observats i serien necessaris estudis més exhaustius del moment d'aplicació de la soca EPS62e per demostrar la implicació d'aquest mecanisme en el biocontrol del foc bacterià.

La soca EPS62e també va mostrar l'habilitat per colonitzar i sobreviure en ferides produïdes en fruits immadurs de poma i pera, així com en flors senceres en condicions ambientals controlades. La soca va sobreviure al menys durant 10 dies en la superfície de les flors en condicions de camp constituint poblacions estables al voltant de 10^6 ufc per corimbe.

En conclusió, la soca EPS62e és efectiva a concentracions moderades en tractaments preventius pel control del foc bacterià en fruits immadurs, flors i brots de perera sotmesos a condicions ambientals controlades. L'exclusió preventiva del patogen mitjançant la colonització de la superfície, el consum de nutrients i la interacció antagonística cèl·lula-cèl·lula amb el patogen semblen ser els principals mecanismes de biocontrol. Aquestes característiques, juntament amb la seva habilitat per colonitzar, multiplicar-se i sobreviure en la superfície de la planta constitueixen trets interessants per a un desenvolupament efectiu d'aquesta soca com a agent de biocontrol del foc bacterià en condicions comercials.

RESUMEN

El fuego bacteriano, causado por la bacteria *E. amylovora* es una enfermedad muy importante a nivel comercial y económico porque afecta a plantas de la familia de las rosáceas y es especialmente agresiva en manzano (*Pyrus malus*) y peral (*Pyrus communis*), así como en plantas ornamentales (*Crataegus*, *Cotoneaster* o *Pyracantha*). Esta enfermedad está distribuida por todo el mundo en zonas climáticas templadas de América del Norte, Nueva Zelanda, Japón, Israel, Turquía y Europa. En España, el fuego bacteriano fue detectado por primera vez en 1995 en el norte del País (Euzkadi) y más tarde, en nuevos focos aparecidos en otras áreas.

El principal objetivo de esta tesis doctoral fue la selección de agentes de biocontrol del fuego bacteriano. Para su consecución se seleccionó un grupo reducido de cepas de *E. amylovora* y se optimizaron varios potasistemas *ex vivo* e *in planta* para ser utilizados en el proceso de selección de agentes de biocontrol. También, se analizó la existencia de rasgos o patrones específicos de los antagonistas asociados a la inhibición de las infecciones causadas por *E. amylovora* y los posibles mecanismos de acción involucrados en el biocontrol de *E. amylovora* por una de las mejores cepas.

En el presente estudio se caracterizaron 53 cepas de *E. amylovora* de diferentes orígenes (localización geográfica y planta huésped) en relación con el tipo de colonia en diferentes medios de cultivo, detección por PCR y ELISA, perfiles metabólicos utilizando sistemas API y Biolog GN, patogénesis en peras inmaduras y diversidad genética mediante RFLPs del gen *ams* y del ADN genómico. Las cepas de *E. amylovora* se mostraron muy homogéneas para todas estas características. Sin embargo, se observaron diferencias principalmente en los perfiles metabólicos, MRFLPs del ADN genómico y patogénesis en peras inmaduras. La variación en los MRFLPs del ADN genómico se relacionó con la distribución geográfica, mientras que los perfiles de utilización de fuentes de carbono parecían estar relacionados con la planta huésped por la tendencia a agrupar las cepas en 2 grupos, uno compuesto principalmente por cepas aisladas de frutales y el otro por cepas aisladas de plantas ornamentales. Se observó una mayor diversidad entre cepas a nivel de virulencia en peras inmaduras. Estudios basados en la relación dosis-enfermedad y en curvas de progresión de la enfermedad permitieron la clasificación de las cepas en función de su agresividad (dosis media efectiva, incidencia máxima de la enfermedad, tasa de progresión de la enfermedad), infectividad (dosis infectiva mínima, momento de inicio de la infección) o de su virulencia en términos generales (Índice de virulencia compuesto, área bajo la curva de progresión de la enfermedad). Las cepas que presentaron la mayor virulencia fueron EPS101, UPN513, UPN544, USV1043, USV4512 y USV4576.

De acuerdo con estos resultados, las cepas CUCM273 y EPS101 de *E. amylovora* fueron elegidas para realizar el proceso de selección de agentes de biocontrol. La cepa EPS101 fue

seleccionada por proceder de nuestra zona de influencia, en Lleida, y porque presentaba las características comunes de la mayoría de cepas de *E. amylovora*. Por el contrario, la cepa CUCM273 fue aislada en Estados Unidos y se seleccionó por ser probablemente una de las cepas más divergente ya que difiere del resto en el serotipo y en el tipo de colonia (blanca, abombada, brillante y mucosa).

El segundo objetivo fue la optimización de los patosistemas que se utilizarían posteriormente en la selección de agentes de biocontrol del fuego bacteriano y en los estudios de interacción consistiendo en ensayos de infección en frutos inmaduros, flores y brotes. Se probaron diferentes métodos de inoculación y concentraciones del patógeno para cada ensayo. Los métodos optimizados consistieron en la inoculación del patógeno mediante la deposición de una suspensión calibrada en las heridas producidas en los frutos inmaduros y en las hojas, en el ensayo en brotes, o en la superficie del hipantio de las flores en el ensayo en flores.

El tercer objetivo fue la selección de agentes de biocontrol. Por este motivo, se probaron alrededor de 533 aislados, principalmente de las especies *Pseudomonas fluorescens* y *Erwinia herbicola* (sinónimo de *Pantoea agglomerans*), obtenidos de flores, frutos y hojas de plantas rosáceas, usando el ensayo en peras inmaduras. Diez aislados seleccionados en este ensayo fueron posteriormente evaluados en el control de las infecciones causadas por *E. amylovora* en flores y brotes en condiciones ambientales controladas. La cepa EPS62e redujo consistentemente las infecciones en flores y brotes presentando unos niveles de control equivalentes a los obtenidos mediante el tratamiento con productos bactericidas químicos.

Con la finalidad de determinar si existían rasgos o patrones característicos presentados por los antagonistas, asociados a la inhibición de las infecciones causadas por *E. amylovora* en peras inmaduras, se caracterizaron las cepas más eficaces utilizando el sistema API, antagonismo *in vitro* contra diferentes patógenos de plantas, producción de compuestos interesantes (antibióticos, sideróforos, quitinasas, ácido indolacético), nivel de inhibición de las infeccions en peras inmaduras, reacción hipersensible y actividad nucleadora de hielo. Se estudiaron las asociaciones entre las características de las cepas y su capacidad de biocontrol mediante el análisis de correspondencias. No se observaron relaciones entre la eficacia en la inhibición de infecciones causadas por *E. amylovora* en peras inmaduras y las características de las cepas.

La cepa EPS62e se identificó como *P. fluorescens*, no produjo ningún antibiótico conocido ni cianhídrico, ácido inolacético, o quitinasas. Tampoco presentó actividad nucleadora de hielo ni indujo la reacción hipersensible en tabaco. La cepa fue tolerante a fungicidas e insecticidas, ligeramente tolerante a kasugamicina y estreptomicina, aunque fue sensible a fosetil de aluminio y a biocidas de amplio espectro como sulfato de cobre u oxicloruro de cobre. Esta cepa presentó un limitado espectro de actividad inhibidora *in vitro* contra patógenos bacterianos de plantas y contra la mayor parte de cepas de *E. amylovora*, que se atribuyó predominantemente a la producción de sideróforos. Sin embargo presentó un amplio espectro de actividad en ensayos *ex vivo* de inhibición de infecciones por cepas de *E. amylovora* en frutos inmaduros. La cepa EPS62e presentó un patrón electroforético

característico de fragmentos de macrorestricción del ADN genómico con el enzima *Swa* I. Este patrón permitió diferenciarla de otros aislados, cepas tipo y agentes de biocontrol de la misma especie.

También se estudió la influencia de distintas concentraciones de la cepa EPS62e y del patógeno sobre el control del fuego bacteriano. La relación entre dosis efectiva media del agente de biocontrol respecto a la de *E. amylovora* (K_z/K_x) se utilizó para comparar la eficiencia de control de la cepa EPS62e en diferentes variedades de peral y órganos de la planta. En función de este parámetro, la mayor eficiencia en la inhibición de infecciones de *E. amylovora* se obtuvo en frutos inmaduros de la variedad Conference en que una célula de la cepa EPS62e fue capaz de inactivar veinte células de *E. amylovora*. La cepa EPS62e también fue altamente eficaz en peras inmaduras de las variedades Passe Crassane y Blanquilla con relaciones de 1 y 40, respectivamente. Por el contrario, fue poco eficaz en flores y frutos de la variedad Doyenne du Comice, con una relación de 10^3, y también en flores de la variedad Conference, con valores de 570.

El último objetivo del presente estudio fue la determinación de los mecanismos de acción involucrados en el biocontrol de *E. amylovora* mediante EPS62e. La antibiosis fue descartada porque la cepa no produjo ninguno de los antibióticos descritos en *P. fluorescens* ni inhibió a la mayoría de las bacterias fitopatógenas ni cepas de *E. amylovora* en ensayos de difusión *in vitro* en diferentes medios de cultivo. Contrariamente, se observó que era necesaria la interacción directa en el proceso de inhibición mediante el contacto célula-célula. Los estudios de interacción realizados a distintas concentraciones de ambas bacterias y relaciones de la cepa EPS62e y EPS101 permitieron estimar la tasa de crecimiento relativo de *E. amylovora* en función del incremento de la relación EPS62e:*E. amylovora*. Se observó que este parámetro era útil para comparar la eficacia de las cepas antagonistas.

Se obtuvieron evidencias de la implicación de la competencia por nutrientes al observar una leve inhibición del crecimiento de *E. amylovora* cuando la cepa EPS62e y *E. amylovora* estaban físicamente separadas por una membrana semipermeable, pero sólo en zumo de pera diluido o cuando la relación EPS62e:*E. amylovora* era muy alta. Además, la cepa EPS62e exhibió una buena habilidad en la competencia por nutrientes porque presentó una mayor velocidad máxima de crecimiento (μ_{max}) y afinidad por nutrientes (menor K_s) que *E. amylovora* en zumo de peras inmaduras, así como un alto índice de solapamiento de nicho (*NOI*), demostrando que es capaz de utilizar la mayor parte de las fuentes de carbono utilizadas por *E. amylovora*.

La implicación de la inducción de respuesta defensiva en la planta se evaluó como posible mecanismo en la inhibición de *E. amylovora* mediante la cepa EPS62e. En este caso, la cepa mostró la capacidad de retrasar la aparición de infecciones causadas por *E. amylovora* en plantas dónde previamente se había infiltrado en el mesófilo de las hojas. Sin embargo, se observó un efecto inconsistente de los resultados y son necesarios estudios más exhaustivos del momento de aplicación de la cepa EPS62e para demostrar la implicación de este mecanismo en el biocontrol del fuego bacteriano.

La cepa EPS62e también mostró habilidad para colonizar y sobrevivir correctamente en heridas producidas en frutos inmaduros de manzana y pera, así como en flores enteras en condiciones ambientales controladas. La cepa sobrevivió durante al menos 10 días en la superficie de las flores en condiciones de campo constituyendo poblaciones estables de alrededor de 10^6 unidades formadoras de colonia por corimbo.

En conclusión, la cepa EPS62e es efectiva a concentraciones moderadas en tratamientos preventivos para el control del fuego bacteriano en frutos inmaduros, flores y brotes de peral sometidos a condiciones ambientales controladas. La exclusión preventiva del patógeno mediante la colonización de la superficie, el consumo de nutrientes y la interacción antagonística célula-célula con el patógeno parecen ser los principales mecanismos de biocontrol. Dichas características, juntamente con su habilidad para colonizar, multiplicarse y sobrevivir en la superficie de la planta constituyen rasgos interesantes para un desarrollo efectivo de esta cepa como agente de biocontrol del fuego bacteriano en condiciones comerciales.

SUMMARY

Fire blight, caused by *E. amylovora* is a serious disease of rosaceous plants that is especially destructive to apple (*Pyrus malus*) and pear (*Pyrus communis*), but also to ornamental plants (*Crataegus*, *Cotoneaster* or *Pyracantha*), with great commercial and economic interest. The disease is distributed over the world in temperate climate areas including North America, New Zealand, Japan, Israel, Turkey and Europe. In Spain, fire blight was first detected in 1995 in the North of the country (Euzkadi) and later, several new outbreaks have appeared in other areas.

The main objective of this PhD thesis was to select potential biocontrol agents of fire blight, for this reason a reduced group of strains of *E. amylovora* was selected and *ex vivo* and *in planta* model pathosystems were optimized to carry out the screening of potential biocontrol agents. Also, the existence of specific traits or patterns displayed by the antagonists associated to inhibition of *E. amylovora* infections and the putative mechanisms of action involved in the biocontrol of *E. amylovora* by one of the best strains were studied.

A group of strains of *E. amylovora* was selected to carry out the screening of potential biocontrol agents of fire blight. In the present study 53 strains of *E. amylovora* from different origins (geographical location and plant host) were characterized attending to cultural characteristics, detection by PCR and ELISA, metabolic profiles based on API and Biolog GN systems, pathogenicity in immature pear fruits and genetic diversity by RFLPs of *ams* gene and genomic DNA. *E. amylovora* strains appeared very homogeneous. Nevertheless, some diversity was observed mainly in metabolic profiles, MRFLPs of genomic DNA and pathogenicity on immature pear fruits. The variation on MRFLPs of genomic DNA was related to geographical distribution. Whereas, variation on carbon source use profiles seems to be related to plant host of isolation with a trend to group strains in two clusters, one mainly composed of strains isolated from pome fruit trees and another of strains isolated from ornamental plants. Greater diversity was observed on virulence of strains on immature pear fruits. Studies based on dose-disease relationships and disease progression curves allowed the classification of strains according to aggressiveness (median effective dose, maximum disease incidence, rate of disease progression), infectivity (minimum infective dose, time of start of infection), or general virulence (composite virulence index, area under disease progression curve). The strains that presented the highest virulence were EPS101, UPN513, UPN544, USV1043, USV4512 and USV4576.

Accordingly, two strains of *E. amylovora* were selected for the screening of potential biocontrol agents of fire blight: EPS101 and CUCM273. EPS101 was selected because it was isolated from our influence region, in Lleida, and presented the common characteristics of most *E. amylovora* strains. In contrast, CUCM273 was selected because it was isolated in

USA and it is probably the most divergent strain differing in serotype, and did not present the white, domed, shiny, mucoid colonies as the typical strains.

The second objective was the optimization of model pathosystems to be used in the selection of biocontrol agents of fire blight and interaction studies. Immature pear fruit, blossom and shoot infection assays were optimized. Different inoculation methods and pathogen concentrations were tested for each assay. Finally, the optimized methods consisted of pathogen inoculations by deposition of a calibrated suspension on wounds produced in the leaves in the shoot assay and in immature fruits or on the hypanthium surface of flowers in the blossom assay.

The third objective was the selection of potential biocontrol agents. For these purpose, around 533 isolates, mainly composed of *Pseudomonas fluorescens* and *Erwinia herbicola* species, obtained from flowers, fruits and leaves of Rosaceous plants, were screened as potential biocontrol agents of fire blight using an immature pear fruit assay. A selection of 10 isolates was subsequently retested for control of blossom and shoot infections under controlled environment conditions. Strain EPS62e consistently reduced blossom and shoot blight providing control levels equivalent to that provided by chemical agents.

In order to find the existence of specific traits or patterns displayed by the antagonists associated to inhibition of *E. amylovora* infections in immature pear fruits, the most efficient strains were characterized using the API system, *in vitro* antagonism on agar media against different plant pathogens, production of interesting compounds (antibiotics, siderophores, chitinases, indoleacetic acid), level of inhibition of infections in immature pear fruits, hypersensitivity reaction and ice nucleation activity. Associations among the traits in the strains were studied by correspondence analysis. No relationships were observed between the efficacy in the inhibition of infections caused by *E. amylovora* in immature pear fruits and strain characteristics.

Strain EPS62e was identified as *P. fluorescens,* does not produce known antibiotics nor other compounds like cyanide, indolacetic acid, and chitinases, neither it presents ice nucleation activity nor induce HR on tobacco. The strain is tolerant to fungicides and insecticides, quite tolerant to kasugamycin and streptomycin, although it is sensible to fosetyl-aluminium and to wide-range action biocides like copper sulphate and copper oxychloride. It presents a very limited spectrum of *in vitro* activity against bacterial plant pathogens and *E. amylovora* strains predominantly mediated by siderophores, but a broad range of activity in *ex vivo* assays against *E. amylovora* strains on immature pear fruits. Strain EPS62e exhibits a characteristic electrophoretic pattern of macrofragments of digestion of genomic DNA with *Swa* I. This is a differential characteristic from other isolates, type strains and biocontrol agents of the same species.

In the present study, the influence of EPS62e and pathogen concentrations on fire blight control was also studied. The median effective dose ratio was used to compare the efficiency of EPS62e among cultivars and plant materials. According to this parameter, the highest efficiency in the inhibition of *E. amylovora* infections was obtained in immature pear fruits of

Conference cultivar. In this plant material one cell of EPS62e was capable to counteract twenty cells of *E. amylovora*. EPS62e was also highly effective in immature pear fruits of Passe Crassane and Blanquilla cultivars with ratios of 1:1 and 40:1, respectively. In contrast, EPS62e was less efficient in fruits and flowers of Doyenne du Comice cultivar, with a median effective dose ratio around 10^3, and in flowers of Conference cultivar, with values of 570.

Another objective of the present work was to determine the putative mechanisms of action involved in the biocontrol of *E. amylovora* by EPS62e. The implication of antibiosis was excluded because the strain did not produce the antibiotics described for *P. fluorescens* neither inhibited in the *in vitro* agar test neither most of bacterial phytopathogens nor *E. amylovora* strains using different types of media. Contrarily, it was observed that direct interaction is involved in the inhibition process because cell-to-cell contact is needed for the inhibition of *E. amylovora*. Interaction studies performed at increasing concentrations and ratios of EPS62e and EPS101 allowed to estimate the rate of change (r) of the relative growth of *E. amylovora* related to the increase in the ratio EPS62e:*E. amylovora*. This parameter was found useful to compare the efficacy of antagonistic strains.

Evidence that nutrient competition is involved in the inhibition was also obtained because of a slight inhibition of growth of *E. amylovora* was observed when EPS62e and *E. amylovora* were physically separated through a semipermeable membrane, but only in diluted pear juice or at high EPS62e:*E. amylovora* ratios. In addition, EPS62e exhibited a good nutrient competition ability because it presented a greater maximum growth rate (μ_{max}), and higher nutrient affinity (lower K_s) than *E. amylovora* on immature pear fruit juice, and a high *NOI*, demonstrating that is capable to use most carbon sources used by *E. amylovora*.

The involvement of induction of plant defense responses was also evaluated as a possible mechanism in the inhibition of *E. amylovora* by EPS62e. EPS62e showed the capacity to delay fire blight infections upon being infiltrated in the mesophyll of leaves of pear plants. However, an inconsistent effect was observed and more exhaustive studies focused on the timing of application of EPS62e are required to prove the involvement of this mechanism in biocontrol of fire blight.

EPS62e also showed ability to colonize and survive well in wounds of immature apple and pear fruits and in intact flowers under controlled environment conditions. The strain showed the capacity to colonize and survive almost during ten days onto the flowers surface under field conditions at stable population levels around 10^6 cfu·corymb^{-1}.

In conclusion, the strain EPS62e was effective at moderately concentrations in preventive treatments for control of fire blight in immature fruits, flowers and shoots of pear under controlled environment conditions. The preemptive exclusion of the pathogen by plant surface colonization and nutrients depletion, and cell-to-cell antagonistic interaction with pathogen cells appear to be the main mechanisms of biocontrol. Its ability to colonize, rapidly grow and survival in the plant surface constitute interesting traits for an effective development as a fire blight biological control agent under commercial conditions.

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ABBREVIATIONS

a.i.	Active ingredient
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
ARDREA	Amplified ribosomal DNA restriction analysis
AUDPC	Area under disease progression curve
BRS	Billing's revised system
BSA	Bovine serum albumin
cfu	Colony-forming units
CHEF	Contour-clamped homogeneous electric field
cv.	Cultivar
CVI	Composite virulence index
DASI	Double antibody sandwich direct
dsp	Disease-specific
ED_{50}	Median effective dose
ELISA	Enzyme linked immunosorbent assay
EPPO	European and Mediterranean Plant Protection Organization
EPS	Exopolisaccharides
GMO	Genetically modified organism
hrp	Hypersensitive reaction and pathogenesis
IGS	Intergenic spacer
ISR	Induced systemic resistance
KB	King's B
MAPA	Ministerio de agricultura, pesca y alimentación
MID	Minimum infective dose
NOI	Niche overlap index
OD	Optical density
PCA	Phenazine-1-carboxilic acid
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PGPR	Plant growth promoting rhizobacteria
PhI	2,4-diacetylphloroglucinol
PR	Pathogenesis-related
Prn	Pyrrolnitrin
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SAR	Systemic acquired resistance
SAS	Statistical analysis system
SEM	Scanning electronic microscopy
SI	Severity index
SNA	Sucrose nutrient agar
U	Units

CHAPTER 1

Introduction

FIRE BLIGHT DISEASE

The main losses in fruit production of pears and apples are due to bacterial and fungal diseases, particularly fire blight (*Erwinia amylovora*) (*Figure 1.1-A*), brown spot (*Stemphyllium vesicarium*) (*Figure 1.1-B*), bacterial necrosis (*Pseudomonas syringae*) (*Figure 1.1-C*), and scab (*Venturia* spp.) (*Figure 1.1-D*).

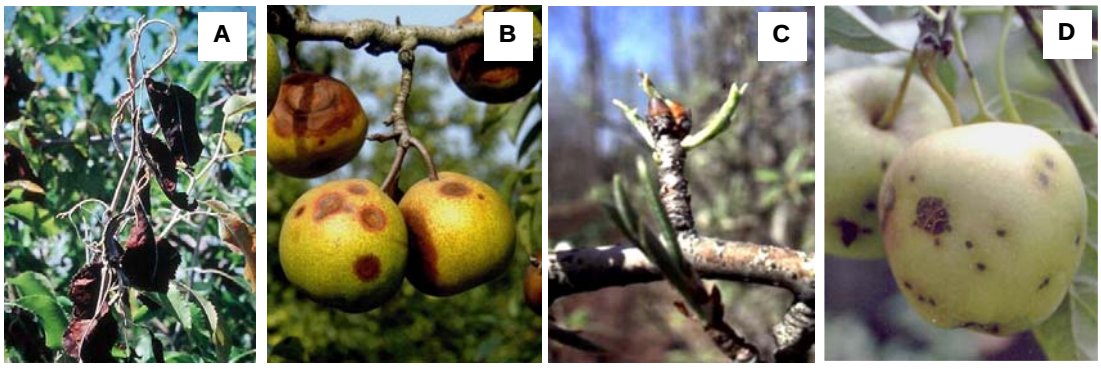

Figure 1.1. Bacterial and fungal diseases on pear and apple: Fire blight (**A**), brown spot (Photo of E. Montesinos) (**B**), bacterial necrosis (Photo of E. Montesinos) (**C**), pear scab (Photo of F. Ortega) (**D**).

Fire blight is caused by bacterium *E. amylovora*, a Gram-negative rod belonging to the Enterobacteriaceae. The disease consists of a necrosis that affects most of the species of Pomoideae and some species of other subfamilies like Rosoideae (Starr *et al*., 1951; Evans, 1996) and Amygdaloideae (Mohan and Thompson, 1996), but it is especially destructive in apple (*Pyrus malus*) and pear (*Pyrus communis*).

Fire blight is probably the most serious disease affecting pear and apple cultivars in many countries. This bacterial disease causes great economical losses in many European and Mediterranean countries and in USA where most of commercial orchards of pear and apple

trees are sensible to fire blight. The severity and the huge economic impact of these outbreaks are a consequence of the virulence of *E. amylovora* and of its ability to survive as endophyte or epiphyte for variable periods of time depending on environmental factors (Thompson, 2000).

In apple and pear trees fire blight produces severe damage or death in the nursery and in the orchard, delay of bearing in young trees due to frequent blighting of shoots and limbs, loss of limbs or entire trees older plantings as the result of girdling by cankers, and partial loss of crop by the blighting of the blossoms and young fruits. Although, disease damage can vary from one year to another, from one orchard to another or from a region to another depending on individual orchard factors and weather conditions.

In Spain the extension of apple and pear is about 71,785 ha with an annual production of 1,368,900 tons, representing around 13.2% of apple and pear production in Europe (MAPA, 2003). Besides, most of pear and apple cultivars present in Spain are sensible to fire blight. The nursery activity related to the production of fruit and ornamental plants in many cases sensible to fire blight is another important sector affected by the introduction of fire blight. Fire blight is especially dangerous in production regions where most of pear and apple cultivars are sensible. Spreading of fire blight into Catalunya could produce a great impact due to the economical importance of apple and pear production, because there are 15,292 ha of apple and 13,623 ha of pear orchards (MAPA, 2003).

1. The pathogen

E. amylovora produces gram-negative rod shaped cells of about 0.3 µm x 1 µm in size, motile by peritrichous flagella, facultative aerobic, non-sporulated, that belong to the Enterobacteriaceae and is strictly associated with plants (Brenner, 1984).

1.1. Cultural and morphological characteristics

Colony morphology depends strongly upon the culture media and growth conditions used. Characteristic growth on different media has been described (*Table 1.1*). Selective media were developed to allow an easier isolation, and also to give a typical aspect to *E. amylovora* colonies, increasing the selectivity of the medium. Some differences in the colony morphology have been observed on certain media between isolates of *E. amylovora*, but these morphological differences are not linked to any known characteristic in physiology or pathogenicity (Paulin and Samson, 1973). A cultural characteristic of *E. amylovora* strains is the formation of colonies on 5% sucrose nutrient agar (SNA) typically white, domed, shiny, mucoid (levan type) after 2 or 3 days at 27 ºC. Non-levan forms are rarely isolated.

Table 1.1. Colony characteristics of *E. amylovora* on differential and selective media (Paulin, 2000)

Medium[x]	**Medium type**[y]	**Colony morphology and colour**	**Reference**
SNA	D	Domed, circular, mucoid	Billing *et al*. (1960)
D3	S	Clear red coloration of the medium	Kado and Heskett (1979)
MS	S	Red to orange colour	Miller and Schroth (1972)
KB	D	White, circular, mucoid	Paulin and Samson (1973)
CG	S	Craters on surface of colonies	Crosse and Goodman (1973)
TTN	S	Chalky white, entire, smooth glossy surface, doghnut-shaped	Ritchie and Klos (1978)
CCT	S	Smooth, large, pulvinate, light blue opalescent, with craters	Ishimaru and Klos (1984)
MM_2 Cu	D	Yellow, circular, smooth	Bereswill *et al*. (1997)

[x]SNA, sucrose nutritive agar; MS, Miller-Schroth medium; KB, King's B medium.
[y]D, diagnostic medium; S, selective medium.

E. amylovora is capable to growth between 3–5 ºC and 37 ºC, being the optimal temperature 25-37 ºC (Billing *et al*., 1961). The bacterial cells resistance to high temperatures was studied, in an attempt to propose a technique to get free plant material of endophytic populations of *E. amylovora* by heat treatment (Aldwinckle and Gustatson, 1993). It was found that a temperature of 45 ºC for 70 min or 50 ºC for 50 min was enough to inactive pure cultures of *E. amylovora*. Some variations were noted between strains (Keck *et al*., 1995).

Growth factors required by *E. amylovora* on laboratory culture media are nicotinic acid (Starr and Mandel, 1950) and thiamine. Thiamine is required by a few wild-type strains (Bennett and Billing, 1978) and strains cured of the pEA29 plasmid. The need of nicotinic acid is not a common requirement among species of the genera *Erwinia* and it was proposed as a biochemical test for *E. amylovora* characterization (Holt *et al*., 1994).

1.2. Serology

E. amylovora is serologically homogeneous and has few agglutinogens in common with related species and saprophytes found in plants. Serology has been used to diagnose, improve the description of the species, and study the homogeneity and differentiation of species from other supposed closely related species. Diagnosis and detection by serological techniques has been accepted internationally.

There are several techniques based on the serology of *E. amylovora* that are used with different purposes for detection, monitoring the bacterial population on the plant surfaces or histological studies of the infection process. Most of these techniques are focused to the detection of *E. amylovora* in symptomatic and asymptomatic tissues. Double Antibody Sandwich Indirect (DASI) Enzyme-Linked Immunosorbent Assay (ELISA) has been further proposed in association with enrichment on suitable media for a specific and sensitive detection of *E. amylovora* (Gorris *et al*., 1996).

1.3. Host range, pathogenicity and strain diversity

Several studies have shown a great homogeneity between strains of *E. amylovora*. A great homogeneity of host range, genetic diversity and pathogenicity has also been observed between the strains. Only some differences have been found in virulence, colony morphology or serology, mainly related to the origin of the strains.

Recent studies based on molecular techniques have greatly advanced our knowledge of genetic diversity and pathogenicity within *E. amylovora* strains, providing tools to characterize strain diversity and pathogenicity (Vanneste *et al*., 1990; Mcmanus and Jones, 1995; Zhang and Geider, 1997).

1.3.1. Host range

E. amylovora is known as a pathogen with wide host range affecting Rosaceae plants. Fire blight has been described on approximately 200 species in 40 Rosaceous genera (van der Zwet and Keil, 1979). Among pome fruits the most affected genera are *Pyrus*, *Malus*, *Cydonia* and *Eriobotrya*. Many ornamental plants are hosts of *E. amylovora* like species of the genera *Crataegus*, *Cotoneaster*, *Pyracantha*, *Chaenomeles*, *Photinia* and *Sorbus* (van der Zwet and Beer, 1995).

The sensibility to the pathogen and the severity of the infections change considerably with host being correlated with the species or cultivar in pome fruit (*Table 1.2*) and in ornamental plants (*Table 1.3*) (Le Lézec *et al*., 1997; van der Zwet and Beer, 1995).

In general, *E. amylovora* is not host species-specific. Most of the strains of *E. amylovora* are pathogenic on different plant hosts. However strains that are host species-specific have also been described. Even so, pathovars based on pathogenicity to specific host have not been described.

It was also observed that degree of virulence is a differential characteristic among the strains of *E. amylovora*. Virulence can differ depending on the cultivar, plant organ and physiological state of the plant. Besides, a marked variability between the virulence of some strains was noted and correlated with morphological and physiological characteristics (Ark, 1937).

Table.1.2. Susceptibility of apple (*Pyrus malus*) and pear (*Pyrus communis*) to fire blight (Boyd and Jacobi, 2000)

Host	**Degree of Susceptibility**		
	Extreme	**Moderate**	**Low or resistant**
Apple	Braeburn Fuji Gala Jonathan Lodi Rome Beauty Yellow transparent	Early McIntosh Granny Smith Grimes Golden Golden Delicious Jonafree Jonagold Missouri Pippin Royal Gala Sharon Summer Red	Golden Gem Red Delicious Winesap Haralson Liberty Prima Priscilla Redfree Top Red
Pear	Abate Fetel Aurora Barlett Blanquilla Bosc Clapp's Favorite Comice Conference General Leclerc Max-Red Barlett Passa Crassana Starkrimson Winter Nallis	Anjou Dawn Douglas Kieffer Seckel Sparklett	Harrow Delight Magness Maxine Moonglow Starking Delicious

Table 1.3. Susceptibility of ornamental plants to fire blight depending of species (Van der Zwet and Beer, 1995).

Genera	**Degree of Susceptibility**		
	Extreme	**Moderate**	**Low or resistant**
Cotoneaster	*bullatus* *francheti* *lacteus* *lucidus* *multiflorus* *reticulatus* *salicifolius* *simonsii* *watereri*	*apiculatus* *divaricatus* *foveolatus* *integerrimus* *nitens* *sternianus*	*amoenus* *adpressus* *canadensis* *dammeri* *horizontalis* *microphyllus* *praecox* *zabelli*
Crataegus	*alemanniensis* *monogyna* *oxycantha* *pentagyna*	*lavallei* *carrierei*	*arnoldiana* *coccinea* *douglasii* *phaenopyrum* *prunifolia* *punctata* *viridis*
Pyracantha	*angustifolia* *atalantioides* *koidzumii*	*coccinea* *rogersiana*	several hibrids
Sorbus	*aria*		*aucuparia* *intermedia*

1.3.2. Pathogenicity

The development of new molecular techniques has allowed finding two important factors, genes termed *hrp* (hypersensitive reaction and pathogenicity) and *ams* (amylovoran), involved in pathogenesis of *E. amylovora* (Vanneste *et al*., 1990; Bellemann and Geider, 1992).

The identification and localization of a *hrp* gene cluster has provided a tool to understand the pathogenicity of *E. amylovora*. Products of *hrp* genes can be classified into three categories based on their functions in pathogenesis: regulatory, secretory and secreted. Regulatory proteins control the expression of other *hrp* genes; secretory proteins, many of them structural components of a protein secretion apparatus, are involved in secreting target proteins to the medium; and secreted proteins are delivered through the secretion apparatus. Secreted proteins include harpins and potential effector proteins, which are likely to directly affect host metabolism and promote parasitism (Kim and Beer, 1998).

Exopolisaccharides (EPS) have multiple roles during the colonization of host plants by this pathogen. Moreover, encapsulated bacteria survive better under dry conditions and are more resistant to oxidative stress. Besides, the best known EPS, the amylovoran, seems to be implicated in the hypersensitivity reaction induced by harpin (Kim and Beer, 1998).

1.3.3. Strain diversity

Several studies have indicated that strains of *E. amylovora* form a homogeneous group (Momol and Aldwinckle, 2000). Homogeneity within the species *E. amylovora* has been recognized when different populations of isolates were studied after introduction of the disease in Europe (Paulin and Samson, 1973) even when a large number of isolates was tested.

Recent development of molecular techniques allow comparisons of the genomic organization of strains and will provide a more complete information of isolates, and of the differences between strains of diverse origins (host plant, geographical) (McManus and Jones, 1995; Beer *et al*., 1996; Momol *et al*., 1997).

The PCR products obtained using primers derived from pEA29 have been used to distinguish several strains of *E. amylovora* depending on plant host. Strains isolated from *Maloideae* plants and most of strains isolated from *Rubus*, produced a 1 Kb PCR product, while strains isolated from Asian pear in Hokkaido Island produced slightly larger bands of sizes from 1.1 to 1.2 Kb (Kim *et al.,* 1995). Studies of Restriction Fragment Length Polymorphism (RFLP) of PCR products showed distinct groups of banding patterns according to the specific geographical area of origin. The banding patterns obtained by *Xba* I digestion and PFGE revealed significant differences among strains from different geographical areas (*Table 1.4*). This technique has been proposed to trace the origin of the pathogen after new outbreaks of fire blight (Zhang *et al.,* 1996).

Table 1.4. PFGE patterns described according to geographical origin

New pattern	Related pattern in strains from	Origin
Pt1	Central Europe, New Zealand	England
Pt2	East Mediterranean regions	Egypt
Pt3	Northern France, Belgium	France
Pt4	Israel	England

In studies of Restriction Fragment Length Polymorphism (RFLP) using *hrp* gene, five different patterns were discernible: one for *Maloideae* strains, two for *Rubus* strains and two for Asian pear strains of Hokkaido Island (Kim *et al.,* 1996).

Random Amplified Polymorphic DNA (RAPD) fragment analysis has been used to group strains upon the host plants or geographical origins from which they were isolated, obtaining the same groups based on the RFLP analysis of *hrp* gene: *Maloideae*, *Rubus* and Hokkaido (Asian pear in Hokkaido Island, Japan) (Momol *et al.,* 1997). The use of Amplified Fragment Length Polymorphism (AFLP) analysis have permitted to increase the number of polymorphic bands produced by PCR fingerprints providing the most useful tool so far for discriminating among strains of *E. amylovora* (Rico *et al*., 2004).

Studies using Amplified Ribosomal DNA Restriction Analysis (ARDREA) (Selenska-Pobell *et al.,* 1998), based on 16S-23S intergenic spacer (IGS) regions amplified and digested with several restriction enzymes, have been used to fingerprint the *E. amylovora* strains isolated in Australia (Kim *et al.,* 1999). Grouping of *E. amylovora* strains based on RAPD (Momol *et al.,* 1997) and ARDREA (Momol *et al.,* 1999) were similar, except for the Hokkaido strains, which did not cluster into the main group defined by ARDREA.

2. Symptoms

E. amylovora can infect any portion of a susceptible plant and disease symptoms are due to the host cell collapse and tissue decompartimentalization resulting in water-soaking and tissue necrosis (Eden-Green and Billing, 1974). The common types of infections are blossom or shoot blight, branch and trunk cankers and rootstock blight. Fire blight symptoms vary from a species or a variety to another, but the most spectacular symptoms are observed in pear trees. Infected pear trees can dead acquiring the typical aspect of burned trees that gives the name to the disease (Boyd and Jacobi, 2000).

Blossom blight is most common on pear, apple, hawthorn, mountain-ash, and *Pyracantha*. Infected blossoms become water-soaked and turn brown. Bacteria may then grow down into

the blossom bearing twigs (spur blight) (*Figure 1.2-A*). Leaves on the spur become blighted, turning brown on apple and black on pear. Although, the most obvious symptom of the disease is the shoot blight phase which first appears one to several weeks after petal fall. Twig blight starts at the growing tips of shoots and moves down into older portions of the twig. Blighted twigs first appear water-soaked, turn dark brown or black.

Blighted leaves remain attached to the dead branches through the summer. The end of the branch may bend over, resembling a shepherd's crook or candy cane (*Figure 1.2-B*). Infected tissue may exude either small droplets of milky-white ooze or fine, hairlike strands containing millions of *E. amylovora* bacteria that can initiate new infections. The ooze, which later turns an amber color, contains bacteria that also are capable of causing new infections (*Figure 1.2-C*).

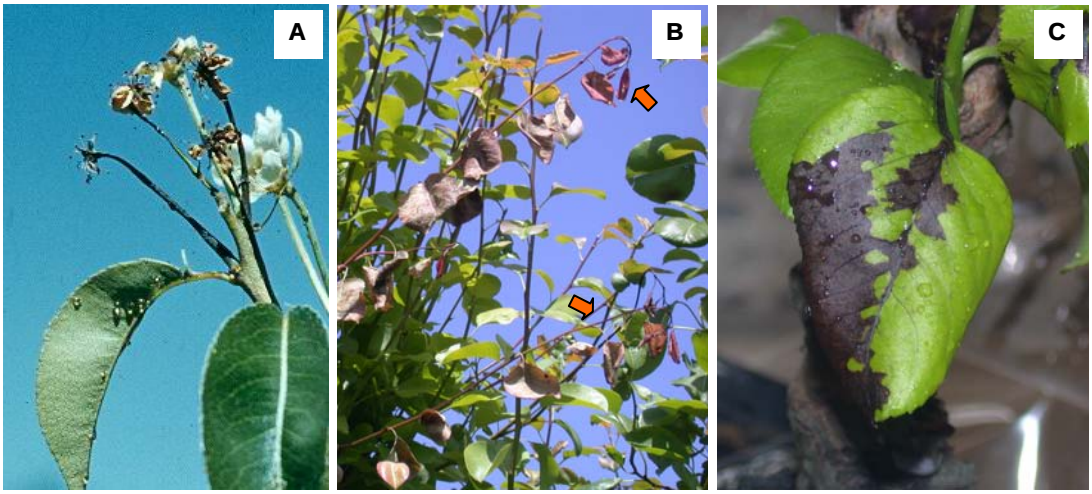

Figure 1.2. Blossom blight on pear (http://www.agf.gov.bc.ca/croplive/croprot/fireblyt.htm) (**A**), shoot blight resembling "Shepherd's crook" (**B**), and necrotic leaf with exudates (**C**).

Fruits are also susceptible to infection until just before maturity. Diseased fruit is first water-soaked, turns brown, shrivels, and turns black (*Figure 1.3-A*). Droplets of milky and sticky bacterial ooze are commonly observed on the fruit surface during wet, humid weather.

The infection may continue grow down into large branch or trunk, forming a canker (*Figure 1.3-B*). Branch and trunk cankers can continue to enlarge resulting in the death of the entire branch or tree. The surface of a canker is somewhat sunken, relative the surrounding healthy tissue, and the bark is usually darker in color.

Fire blight may also spread into the root system, leading to tree death. This phenomenon called rootstock blight usually occurs in high density orchards planted with susceptible rootstock. Rootstock blight is caused by formation of cankers on susceptible rootstocks. These infections can also lead to the invasion of the entire root system and the rapid death of the tree (*Figure 1.3-C*).

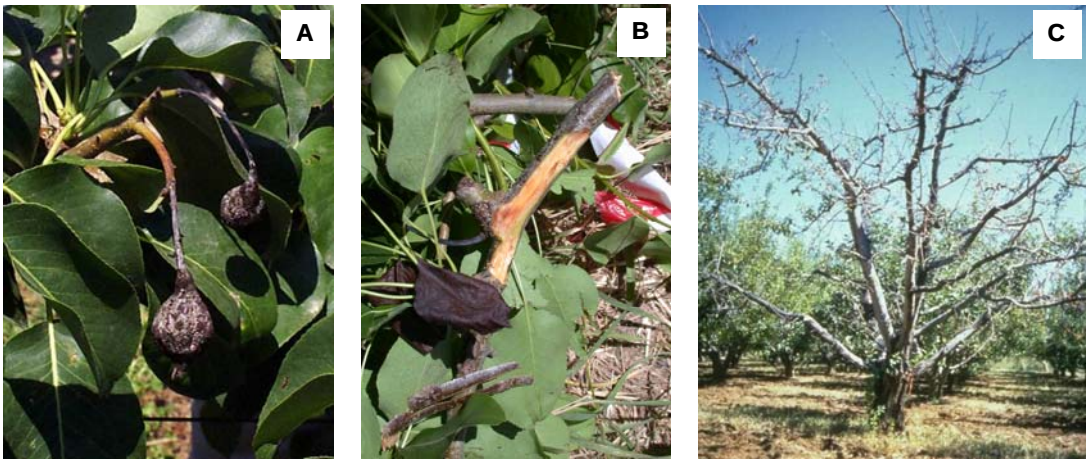

Figure 1.3. Necrotic fruits (**A**), fire blight canker on apple branch (**B**), and dead tree (Photo of J. L. Juan (**C**).

3. Epidemiology and disease cycle

Fire blight is a complex disease where the pathogen is mainly associated with the plant tissue. Infection events occur only when the host is in a susceptible condition, pathogen inoculum level is adequate and environmental conditions are suitable. Disease cycle of fire blight has been described by different authors (van der Zwet and Beer, 1995; Thomson, 2000) (*Figure 1.4*).

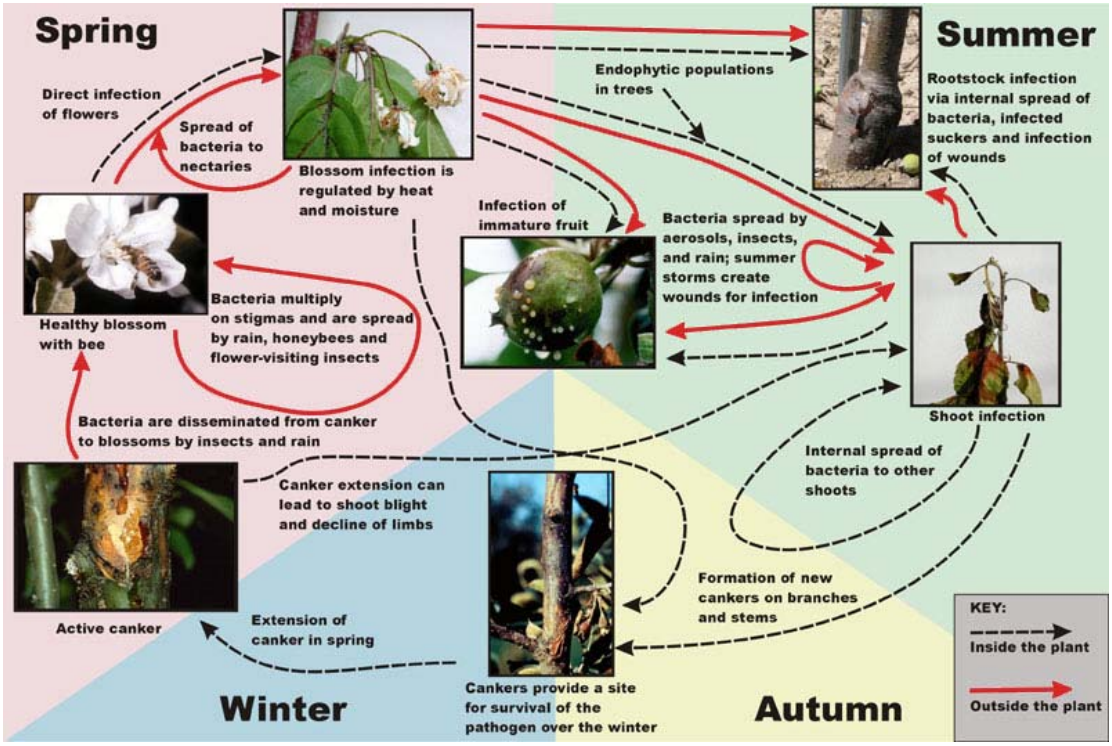

Figure 1.4. The disease cycle of fire blight caused by the bacterium *Erwinia amylovora*. Dashed lines represent movement of bacteria and spread of disease within the plant and solid lines represent movement of bacteria outside the plant. Graphics of Wilbur Hershberger. Modified from Norelli (2003) after Sherman Thomson (Thomson, 2000). (http://www.caf.wvu.edu/kearneysville/disease_descriptions/fire_blight_disease_cycle.htm).

Bacteria overwinter in blighted branches and at the edge of cankers that were formed by infections initiated in previous years. Reactivation of plant growth in the spring, when the temperatures are above 18 ºC, results in the increased nutrient supply to the dormant bacterial population and resurgence of disease. This process is favored by rain, heavy dews and high humidity.

Masses of bacteria are forced through the bark surface, where they form sweet, gummy exudates called bacterial ooze, that constitute the primary inoculum (Johnson and Stockwell, 1998). The droplets of ooze produced on the canker are spread by splashing rain or insects (mostly bees, flies and ants) to open blossoms or to wounded shoots.

Once on the blossom, bacteria multiply rapidly and may invade the tissue through the nectaries (non-cutinized of flowers parts) and move into the branch. All flowers, leaves and fruit above the point of infection may die. The bacteria then spread from blossom to blossom by rain or pollinating insects. The optimum temperature range for blossom blight infection is 18 to 30 ºC.

Young branch tips are frequently infected by bacteria that have been spread from cankers and infected blossoms. The invasion of shoot tips can occur through natural openings, such as lenticels and stomata, but more commonly through wounds created by sucking insects such as aphids, leafhoppers, and tarnished plant bugs, by wind whipping, by hail or by pruning. Droplets of ooze may form on the shoots and ooze serves as new source of inoculum for the further spread of the disease constituting the secondary inoculum. Shoots remain highly susceptible to infection until vegetative growth ceases and the terminal bud forms. Cankers eventually develop from branch or blossom infections.

During summer and spring, several secondary infection cycles can be developed if the environmental conditions, temperature and humidity are favorable. At the end of vegetative period cankers in trunk and branches can appear constituting the bacterial reservoir during winter. During winter and autumn the pathogen growth stops due to temperature decrease and to the end of vegetative growth.

Warm weather (optimum temperature 24 ºC) and moist favor infection, and rapid tree growth encourages disease development. Nitrogen fertilization, late fertilizer application, poor soil drainage, and other factors that promote succulent growth or delay the hardening of tissues tend to increase the severity of fire blight.

4. Disease distribution

Fire blight was described for first time in 1780 in Hudson Valley of New York State (USA) (Denning, 1794). From New York the disease spread to Canada and to Pacific Coast. In 1919, the disease spread to New Zealand, and in 1957 to Europe through the south of England. The next regions where the fire blight was observed were South-America in 1959 and Egypt and the Netherlands in 1966. Disease was then spread to all Europe, and

nowadays only some countries are considered free from fire blight, and are catalogued as protected zones of fire blight (*Figure 1.5*).

Figure 1.5. Worldwide distribution of fire blight.

E. amylovora was first detected in Spain in late summer 1995 in a field survey in Gipuzkoa (Euzkadi) (*Figure 1.6*). The disease was found in a cider apple orchard close to the Atlantic French border. New outbreaks were discovered in Gipuzkoa (Euzkadi) in 1996 and 1997. In 1996, fire blight was found in *Crataegus* plants in a nursery located in Segovia (Castilla-Leon) and eradicated. No relation with the previous outbreaks was found and the plants infected came from importation. In 1997, several outbreaks were also detected in the North of Navarra, mainly in *Pyracantha*, and an interception of a young tree in Girona province. In 1998, the disease was detected in a commercial nursery in Guadalajara (Castilla-La Mancha). The same year another outbreak was found in a few pear orchards in Lleida (Catalunya) and eradication measures were also taken (Lopez *et al.,* 1999).

Figure 1.6. Distribution of fire blight in Spain.

Active outbreaks newly appeared in Euzkadi, Navarra and Lleida during 1999. In 2000 outbreaks were detected in commercial pear orchards in Zaragoza and Logroño. In 2001 and 2002 the apparition of new foci was relatively less, but in 2003, a reactivation of outbreaks in Zaragoza has been observed. However, the number of outbreaks has not increased exponentially and nowadays disease is quite controlled. Until the moment, in Spain the disease progression has been limited to the North due to the control of the disease by means of the prospection and the eradication of the outbreaks according to the Spanish normative. A last outbreak has been detected in 2003 in a pear orchard in Puigcerda (Girona). Despite several outbreaks of the disease have been detected, fire blight is not yet widespread in Spain and is still considered protected zone.

5. Disease management

Fire blight is a disease difficult to manage because there are no efficient control measures, therefore prevention is the best solution. Because of this reason the EU has considered fire blight as a quarantine pest (EPPO A2 quarantine pest). Disease management is conditioned to the presence or absence of fire blight in the region. If the disease is not introduced in a region and this is considered free of fire blight, all the efforts must be focused to prevent its entry. Basically the strategy consists of using plant material harboring ZP passport and eradicating disease focus by cutting and destruction of infected plant material.

Once the disease has been introduced in a region, apart from eradication measures, the integrated management by the use of several practices must be focused to prevent new infections and to reduce damage. Besides, the low efficacy of control methods makes necessary the combination of different strategies to manage the disease to get a reasonable control, by means of inoculum reduction, limiting the spread, preventing infection with treatments based on chemicals or biological compounds and reducing the susceptibility of the host by cultural practices (Jones and Aldwinckle, 1990; van der Zwet and Beer, 1995).

5.1. Resistant plant selection and transgenic varieties

Varieties of pear, apple, crabapple and specific ornamental plants have different degrees of susceptibility to fire blight. There are several programs to select or develop resistant varieties by means of genetic modification. However, the introduction of disease-resistance genes by conventional breeding methods is very difficult, due to the apple and pear's heterozygosis, long generation time and self-incompatibility (Lespinasse and Aldwinckle, 2000).

The use of biotechnology can overcome these obstacles by introducing resistance genes directly into commercial cultivars without modifying desirable characteristics. Genes for fire blight resistance can be transferred to both apple and pear using genetic engineering, but no transgenic apple or pear cultivars have been commercialized yet. The genes more

extensively used and studied in apple and pear code mainly for antimicrobial compounds like cecropins, attacins and lisozymes (Norelli and Aldwinckle, 2000).

Some transgenic lines of apple have shown a high level of resistance to fire blight in field trials. However, the commercialization of such transgenic plants for the practical benefit of pear and apple growers will be complicated by regulatory requirements and licensing (Norelli and Aldwinckle, 2000).

5.2. Cultural practices

Cultural practices include insect control, disinfection of potential sources of contamination, pruning, irrigation and fertilization. Most of these practices are focused in the reduction of the plant vigorousity which increases the plant susceptibility to fire blight. The objectives of these practices are the reduction of infection risk, the reduction of the incidence and the severity of the infections as well as the control of disease spread. Furthermore, favorable environmental conditions and cultural practices, like the high plant densities or the long life tree orchards which allows the build-up of high pathogen populations, may increase the intensity of the fire blight to destructive levels.

5.3. Heat treatment

Fire blight can cause problems derived of the fact that the pathogen can remain latent in symptomless tissues (McManus and Jones, 1994). Introduction into nursery fields through contaminated bud wood and into orchards can be especially troublesome. It is important to keep nursery material free of *E. amylovora*, because of the potential of not only introducing the pathogen into areas where fire blight is not present, but also introducing new genotypes of the pathogen in areas where the disease is already established like resistant strains to antibiotics as has been reported in the USA. Heat treatment can be useful to ensure the absence of *E. amylovora* in nursery plant materials as well as to ensure the functionality of European Phytosanitary Passport ZP. This method is based on the application of heat for eliminating *E. amylovora* with minimum damage to the plant material (Sobiczewski *et al.*, 1997). However, this method is still in study, but promising results have been obtained (Ruz, 2003).

5.4. Chemical control

Chemical control is oriented to eliminate or inactivate *E. amylovora* before the penetration in the host tissue by destroying the source of inoculum or by protecting potential invasion sites, such as blossoms or wounds. Most of chemicals available are not systemic and have no curative action. Therefore, bactericides should be applied preemptively during the distinct periods of the host life cycle: when the plant is dormant, in bloom and in post-bloom stages.

A large number of chemicals have been tested against fire blight, but only two groups of chemicals control fire blight efficiently, the copper compounds and the antibiotics. In general, copper compounds have an acceptable efficacy in the reduction of disease incidence, but their activity is insufficient especially under high inoculum pressure. Therefore, treatments with copper compounds must be combined with other measures. Besides, copper compounds cause phytotoxicity on some host plants and organs which limits their use to low doses. Nevertheless, different formulations of copper are available with different degrees of efficacy and phytotoxicity.

From all the antibiotics evaluated against fire blight, only streptomycin, oxytetracyclin and kasugamycin have practical value for field applications (van der Zwet and Beer, 1995; Vanneste, 1996). Streptomycin is considered the most effective bactericide against fire blight, with no phytotoxicity problems at the recommended concentrations, but its use in agriculture has been prohibited in many countries mainly due to the development of resistance by *E. amylovora* and the danger of possible transfer of this resistance to human or veterinary pathogens (Loper *et al*., 1991; Stockwell *et al*., 1996). Due to this limitation other antibiotics have appeared to replace streptomycin, and the most promising are the oxytetracyclin and kasugamycin. Although, oxytetracyclin is less effective than streptomycin and has shown inconsistent efficacy (Paulin *et al*., 1987; Aldwinckle and Norelli, 1990). However, kasugamycin is a good alternative because it is registered in some countries, like in Spain, for its use in the control of several bacterial diseases of fruit trees (Ruz, 2003).

There are also some novel compounds that could be used against fire blight, overcoming some of the disadvantages of copper and antibiotics, such as, flumequin or compounds that induce defense responses in the host like fosetyl-aluminium (Fosetyl-Al), harpin, prohexadione-Ca or benzothiadiazole (BTH). Most of these compounds do not produce phytotoxicity and their effectiveness in some cases is comparable to that of streptomycin though results are sometimes inconsistent. For this reason, some of these compounds have been registered for control fire blight (*Table 1.5*).

Table 1.5. Chemical compounds most used in experiments to control fire blight on Pomaceous plants from 1980 to 1996 (from Psallidas and Tsiantos, 2000)

Compound	**Product name**	**Disease type**	**Host**	**Effectiveness** (% control)	**References**
Copper oxychloride	Miezdan 50	Blossom blight*	Pear[a]	55	Sobiczewski and Berczynski (1990)
Kasugamycin	Kasumin 25%	Blossom blight*	*Cotoneaster*[a]	88	Koistra and de Gruyter (1984)
Streptomycin	Agrimycin 17	Blossom blight*	Cider apple[a]	90	Jones and Byrde (1987)
Flumequin	Fructil™	Shoot blight*	Pear[b]	70	Deckers *et al*. (1987)
Fosetyl-aluminium	Aliette™	Blossom blight*	Pear[b]	74	Demir and Gundogdu (1993)

*****Protective sprays. [a]Natural inoculation. [b]Artificial inoculation.

Chemical control of fire blight often gives inconsistent results in field experiments, and may present low efficacy and phytotoxicity in some tissues. This variability is due to factors like the level of pathogen inoculum, moment of application, weather conditions, plant species or cultivar, method of application and physiological state of the host plant. To solve or reduce this variability, the application of bactericides should be combined with other measures, as a part of an integrated program such as cultural measures, biological control as well as fire blight risk assessment models.

5.5. Fire blight forecasting models

The forecasting models integrate many factors like the physiological stage of the plant, weather conditions, basically temperature and humidity, and other variables affecting disease development. These models are working hypothesis based on a combination of knowledge of the disease, speculation and trial and error (Steiner, 1996). Several fire blight risk assessment models have been developed in Europe like the Billing's Revised System (BRS) (Billing, 1992) and Parafeu by INRA-MeteoFrance (Paulin *et al.,* 1994) both based on responses index; and in USA the Maryblyt system (Steiner, 1990) based on cumulated thermal thresholds and Cougarblight model (Smith, 1993) that estimates bacterial growth rate with degree hours based on a specific growth rate curve. Models give valuable guidance on which to identify potential infection periods and improve the timing of sprays avoiding unnecessary treatments. Fire blight forecasting models also revealed a good correlation between predictions and outbreaks actually observed, and permit to elaborate fire blight risk maps to help disease management (Paulin *et al*., 1994; Montesinos and Llorente, 1999).

5.6. Biological control

Biological control of fire blight is based on the capacity of epiphytic bacteria, mainly of *Pseudomonas fluorescens* and *Erwinia herbicola* species (also called *Pantoea agglomerans*) to inhibit the development of *E. amylovora* infections. Since many years, the capacity of plant epiphytic bacteria to produce antibiotic compounds against *E. amylovora* (Vanneste *et al*., 1992; Wodzinski *et al*., 1994) or to inhibit the infections in immature pear fruits (Beer and Rundle 1983, Vanneste *et al*., 1992) is known. Also the control of fire blight in apple and pear trees (Kearns and Hale, 1993) and in ornamental plants (Wilson *et al*., 1992) by means of *P. fluorescens* and *E. herbicola* has been reported.

The advance in biological control of fire blight has been propelled by selection of effective antagonist strains, by enhanced knowledge of the mechanisms by which these strains suppress disease and by increased understanding of the ecology of bacterial epiphytes on plant surfaces (Johnson and Stockwell, 2000). Some products based on biological control agents have been registered for use. BlightBan A506 and BlightBan C9-1 (Plant Health

Technologies, Boise, ID. USA) based on *P. fluorescens* strain A506 (Lindow *et al*., 1996) and *E. herbicola* C9-1 (Ishimaru *et al*., 1988) are available in USA as commercial products for fire blight control. Besides, there are other biocontrol programs under progress mainly focused on development of products based on *E. herbicola* strains (M232A, NL18, 112Y) (Epton *et al*., 1994).

Because the biological control of fire blight is the object of the present study, this topic will be developed more extensively in the next section.

BIOLOGICAL CONTROL OF FIRE BLIGHT

1. Chemical control of fire blight within the new scenario of chemical control of plant diseases

The agricultural production has been increased notably over the last 30 years, mainly as a consequence of the use of chemicals to control crop diseases. Although, the abusive use of chemicals has caused and still causes environmental pollution, accumulation of some toxic and/or carcinogenic residues in the food chain and in water, or the development of resistance in the pathogen (Lumsden *et al*., 1995). Thus, social and political concerns have influenced the practice of crop protection which has been progressively reoriented to a rational use of pesticides and to a reduction of the number of registered active ingredients to those certainly unavoidable, more selective, less toxic and with a lower negative environmental impact (Gullino and Kuijpers., 1994; Ragsdale and Sisler, 1994). Under this objective, the European Union and countries like USA have undertaken regulatory changes in pesticide registration requirements.

The European Union has established the directive 91/414/CEE for harmonization of the register of pesticides which project a reduction for the year 2008 from nearly 900 existing active substances in 1991 to less than 400 (http://europa.eu.int/comm/food), and the requirement of the presentation of a very extensive dossier including toxicological studies in mammals, ecotoxicology, traceability and environmental impact (Montesinos, 2003). The directive undertakes a severe re-registration of old registered products and of the registration for new products. Therefore the apparition of new products is too slow and the availability of active ingredients will be drastically limited (*Table 1.6*).

Apart from the European Union, similar measures have been adopted in USA. For example, the creation in 1994 of the Division of Biopesticides and Contamination Prevention of the Environmental Protection Agency (EPA) with the basic functions of the biopesticides registration, conduct of integrated control and reduction of chemicals use (Hall and Barry, 1995).

Table 1.6. Number of active ingredients currently available in Europe that have been revised or are pending of revision (http://europa.eu.int/comm/food/plant/protection/evaluation/exist_subs_en.htm)

	Number of active ingredients
Total at 2004 (registered until 1992)	876
Revised at date	46
Pending of revision	495
Nondenfendant by manufacturers-Dropped	343

This situation implicates the necessity to develop new strategies in order to control plant diseases because some crops possibly will keep without defense in front of diseases. Thus, under this context of limitation of chemical pesticides, biological control emerges as an alternative or complement to chemicals. However, development and application of biocontrol is limited compared to chemicals.

2. Biological control of plant diseases

Biological control of plant diseases was initiated 70 years ago. As a result several products based on biological agents are available in the market for the control of many plant diseases and pests (*Table 1.7*). Even so, these products are not used currently because chemicals are cheaper, easier to use and with higher efficacy and consistency of control.

Biological control involves the use of beneficial microorganisms that have the capacity to inhibit pathogens and control the disease they cause. Biological control is sustained by beneficial interactions resulting from competition, antagonism and hyperparasitism of certain microorganisms against plant pathogens, insects and weed (Mathre *et al.*, 1999). Actually several microorganisms involved in such processes are the active ingredients of a new generation of microbial pesticides or are the basis for many natural products from microbial origin (e.g. harpins) or of new compounds upon chemical modification (e.g. pyrrolnitrin) (Montesinos, 2003).

Biocontrol often is less effective and consistent than chemical control and successful use of biocontrol requires a greater understanding of the biology of both the disease and its antagonist.

Nowadays, it is difficult to conceive the biocontrol as an alternative of chemical control, but can be viewed as a complementary disease control strategy, where the benefits from its use will be most significant when integrated with cultural practices and rational application of chemicals. Thus, in sustainable agriculture, biocontrol contributes to the suitable protection of crops complementing the available measures of control which must be combined, although partially they have a limited efficacy (Jiménez-Diaz, 1995).

Table 1.7. Microorganisms registered or under development for biocontrol of plant diseases in USA and other countries (http://www.epa.gov/TTpesticides/biopesticides/product_lists)

Active ingredient	**Product**	**Target pathogen**	**Crop**
Agrobacterium radiobacter	Galltrol-A, Nogall, Diegall	*Agrobacterium tumefaciens*	Several
Ampelomyces quisqualis M-10	AQ10	Mildew	Several
Bacillus cereus strain UW85	Several	-----	Cotton
Bacillus sphaericus	Vectolex	Culex mosquitoes	-----
Bacillus subtilis GBO3	Kodiak, Subtilex, Epic	*Rhizoctonia solani, Fusarium, Alternaria, Aspergillus*	Several
Bacillus thuringensis	Several	Many insects	General
Beauveria bassiana	Naturalis, Mycotrol	Many insects	General
Burkholderia cepacia	Blue Circle	*Fusarium, Pythium,* nemathodes	Several
Candida oleophila I-182	Aspire	*Botrytis, Penicillium*	Lemon tree, Grapefruit
Colletotrichum gloeosporioides	Contans WG	*Sclerotinia minor, Sclerotinia sclerotium*	Vegetables, ornamentals, soya
Coniothyrium minitans	Several	Nemathodes	General
Fusarium oxysporum	Biofox C	*Fusarium oxysporum, Fusarium moniliforme*	Basil, carnation, tomato
Gliocladium catenulatum	Primastop BioFungicide Powder	Fungal root pathogens	General
Glicadium virens GL-21	WRC-AP-1	*R. solani, Phytium*	General
Myrothecium verrucaria	Ditera	Nemathodes	General
Phlebia gigantea	Rotstop	*Heterobasidium annosum*	Trees
Phytium oligandrum	Polygrandon	*Phytium ultimum*	Beet
Burkholderia cepacia	Intercept	*R. solani, Fusarium, Phytium*	Corn, cotton, vegetables
Pseudomonas fluorescens A506	Blightban A506, Frostban A506	*Erwinia amylovora*	Several Rosaceous plants
P. syringae L-59-66	Biosave	*Botrytis cinerea, Penicillium spp., Mucor pyroformis, Geotrichum candidum*	Lemon tree, Grapefruit
Ralstonia solanacearum	Pssol	*Ralstonia solanacearum*	Vegetables
Streptomyces griseoviridis	Mycostop	*Fusarium, Alternaria, Phomopsis, Botrytis, Pythium, Phytophthora*	General
Trichoderma harzianum	Several	*Armillaria, Sclerotinia, Phytophthora, Rhizoctonia, Pythium, Fusarium, Verticillium*	Several

2.1. Mechanisms

Major limitation in the use of biocontrol is the knowledge of the control mechanisms developed by biocontrol agents. Mode of action is poorly understood and this may be attributed to the difficulties in studying the complex interactions that take place between the host, pathogen, antagonist and possibly other microorganisms present in the site of interaction. The knowledge of mechanisms involved in the mode of action of an antagonist may be crucial for its successful development and to improve its efficacy.

The mode of action will determine the strategy in disease control, the implication of its application and the improvement of efficacy and consistency. Determination of the action mechanisms needs the knowledge of many aspects such as the dynamics of populations, disease cycle, epidemiology and interactions that take place between the biocontrol agent, pathogen, host and other microorganisms (Handelsman and Parke, 1989).

There are several mechanisms by which a microorganism may limit the growth of another. Most biocontrol agents do not strictly use one control mechanism, and for this reason disease control is the result of the combination of several mechanisms. There are four main mechanisms operating: antibiosis, nutrient and space competition, induction of host resistance, and direct interaction between the antagonist and the pathogen (Whipps, 2001).

Antibiosis is based on the synthesis of compounds such as antibiotics or toxins that kill or have a detrimental effect on the target organism. Antibiotics are compounds with low molecular weight and active at low concentrations provoking growth inhibition (Fravel, 1988). Antibiotics produced by microorganisms are very diverse in molecular structure and mode of action. Usually the same microorganism can produce several antibiotics. One example is *P. fluorescens* CHA0 that produce several compounds with inhibiting activity against different soil pathogens (Keel *et al*., 1989).

Competition for nutrients and space is based on the capacity to out-compete other microorganisms. It consists typically of fungus or bacterium that grow very fast and overwhelm the target organism. Introduction of a biocontrol agent in the plant provokes the beginning of a competition for space and nutrients between the biocontrol agent, pathogen and other microorganisms living in the same environment. Space and nutrients must be limiting to produce the competition and these limitations vary in function of the environment where the interaction takes place. Disease inhibition is produced when the biocontrol agent is a better competitor for nutrients or space than the pathogen. The process of biocontrol with antagonistic microorganisms is based mainly on competitive exclusion or effective colonization of the points of entry of the pathogen (stomata, lenticels or wounds) (Andrews, 1992).

Induction of defense responses in the host is based on the capacity of certain bacteria to induce physiological changes within the plant which provides systemic protection against a broad range of pathogens. This systemic protection is called systemic acquired resistance (SAR) when the induction is mediated by non-pathogenic aerial colonizators while is called induced systemic resistance (ISR) when the elicitation is mediated by rhizobacteria (van Loon *et al*., 1998).

Direct interactions are the processes where the biocontrol agent recognizes and interacts with the pathogen at the same time that interfere in disease development. The antagonist and the pathogen may interact in several different ways, the most extensively studied of which is the parasitism. Parasitism is mediated by inducible hydrolytic enzymes that destroy the cell wall (Lam and Gaffney, 1993). An example of direct interaction is *Pantoea agglomerans* EPS125 against *P. expansum* (Bonaterra *et al*., 2003).

2.2. Biocontrol of aerial plant diseases

In recent years there has been much interest in biological control of plant pathogens. However, practical biological control strategies applicable to aerial plant pathogens have not been forthcoming.

There are several reasons for this lack of success: limitation in our basic knowledge of microbial communities on the leaf surface; few research programs in which biological control agents were identified, intensively tested, and improved; tendency to adopt biocontrol strategies for soilborne pathogens and apply them directly to aerial surfaces; and the biological control agents are often less effective relative to chemical treatments (Cullen and Andrews, 1984).

However, the environment on the leaf is characterized by extreme conditions provoking that biocontrol agents have difficulties to survive and colonize, and resulting in the low effectiveness and consistency of the biocontrol foliar diseases.

2.2.1. Environmental conditions on leaf surface

Extreme temperature and relative humidity fluctuations, low nutrient availability, and intense radiation are common features of the phylloplane environment. These disturbances range from darkness with free moisture, without solar radiation, to severe dry conditions during day that osmotically shock microorganisms with concurrent irradiation (Montesinos *et al*., 2002). Leaf surface is a nutrient depleted environment with few available carbon and nitrogen sources (Kloepper, 2000).

Even so, phyllosphere includes different environments like leaves, buds and flowers. Buds and flowers are environments more favorable to microbial antagonist establishment and survival because they are protected and stable sites. Besides, leaves are present during all the vegetative period in many plants, and are the aerial organs where the interactions

between the biocontrol agent and the pathogen are more frequent. Flowers and buds have a shorter time of life, but frequently are the entry site of the pathogens (Blakeman, 1985).

These characteristics of the aerial plant part suggest that leaf-associated bacteria must possess particular adaptations that allow them to exploit the leaf environment. Knowledge of the nature of these adaptations and their role in the growth and survival of bacteria is critical to an understanding of the ecology of associated bacteria as well as to strategies for disease control (Beattie and Lindow, 1995).

2.2.2. Desirable characteristics of a phytosphere biocontrol agent

The goal of biological control is to establish sufficiently large populations of biocontrol agents in the site of interaction with the pathogen. To achieve this goal, biocontrol agents must survive, colonize and develop the control mechanism in the pathogen infection sites. In many cases the pre-emptive colonization of the ambient is necessary to get a good disease control (Andrews, 1992), but also the capacity to synthesize antimicrobial compounds may play a role in pathogen exclusion.

The best candidates are epiphytic bacteria because are just adapted to live and survive on plant surfaces. These bacteria are naturally adapted to the environmental stresses and for this reason present some characteristics to become biocontrol agents of aerial plant pathogens. A tolerance strategy requires the ability to resist direct exposure to environmental stresses on leaf surfaces, including UV radiation and low water availability. An avoidance strategy requires the ability to seek and/or exploit sites that are protected from these stresses, including endophytic sites. These sites may be on leaf surfaces, such as in depressions and crevices, and substomatal chambers and intercellular spaces. The combination of both strategies permits these bacteria establish stable populations on leaf surface (Beattie and Lindow, 1999).

There are several mechanisms that confer to bacteria the ability to survive and colonize the environment. This may include the synthesis of anchoring structures like exopolysaccharides on leaf surfaces (Beer *et al.,* 1987) which also prevent cells from desiccation and improve growth and survival. These biofilms can concentrate nutrients from dilute sources, provide protection from predators, and shield cells from lytic enzymes, antibiotics, and other inhibitory compounds.

Another mechanism is motility that permits bacterial movement to internal or protected sites. The contribution of motility has been demonstrated by comparing the behavior of non motile mutants of *P. syringae* on leaves to that of the parental strain (Haefele and Lindow, 1987). Chemotaxis toward plant extracts are the most likely reasons that motile cells survived better than non motile cells on plant surfaces.

There are other specific adaptations to counteract environmental stresses, like nutrients limitation, UV and visible radiation protection and osmotic tolerance. Nutrients limitation can be minimized by the ability to metabolize a broad range of nutrients with a high efficiency

improving the competitiveness, as for example producing iron chelators, mainly siderophores, to solve iron limitation. The pigmentation and specific DNA reparation mechanisms may help in the tolerance to UV and visible radiations, while the osmotolerance permits the adaptation to water availability fluctuations.

In addition to this capacity to colonize and to survive in leaf surfaces, the biocontrol agent must develop some direct mechanism to assure their effectiveness against the pathogen. Most biocontrol agents develop antibiosis against the pathogen. Moreover, antibiosis is not always the mechanism, and there are other mechanisms involved in disease control like competition for nutrients and space, or the direct interaction. For example, *E. herbicola* Eh252 suppresses blossom blight caused by *E. amylovora* by means of the blockage of infection sites (Wilson *et al*., 1992).

2.3. *Pseudomonas fluorescens* and *Erwinia herbicola* as biocontrol agents

The epiphytic microbiota of the phyllosphere represents a large source of microorganisms that can be used to obtain biocontrol agents of foliar diseases, especially, bacteria that represent the predominant microbiota on leaves have a high degree of fitness and will be generally good colonists if inoculated onto other leaves of the same plant species.

The microbiota of aerial plant parts is composed of Gram negative bacteria from the species *P. fluorescens*, *E. herbicola*, *P. syringae* and from genera such as *Aerobacter, Xanthomonas* and *Flavobacterium*. Gram positive bacteria are less abundant though bacteria from genera *Clavibacter*, *Bacillus* and *Lactobacillus* can be found (Cook and Baker, 1983). However, only strains mainly of the species *P. fluorescens*, *E. herbicola* and from *Bacillus* have been described as biocontrol agents.

Strains of *P. fluorescens* and *E. herbicola* are good candidates as biocontrol agents of aerial plant diseases because they develop several control mechanisms ranging from antibiotic production to competitive exclusion for nutrients or space (Wilson *et al.,* 1992; Mitchell *et al*., 1996; Wright and Beer, 1996; Stockwell *et al*., 1998).

Strains of *P. fluorescens* are biocontrol agents of several diseases, in part, due to its capacity to synthesize several antimicrobial compounds. Different strains have been described to inhibit diseases caused by fungi in roots and also in aerial plant parts. Some examples of pathogens controlled are *Rhizoctonia solani* (Howell and Stipanovic, 1980), *Fusarium oxysporum* (Scher and Baker, 1982), *Gaeumannomyces graminis* (Weller, 1983), *Phytium* spp., *Thielaviopsis basicola* (Stutz *et al.,* 1986), *Clavibacter michiganensis* (de la Cruz *et al.,* 1992), *Sclerotinia sclerotiorum* (Expert and Digat, 1995) and *Stemphylium vesicarium* (Montesinos *et al*., 1996).

Strains of *E. herbicola* have been described as biocontrol agents against several foliar, root and postharvest phytopathogens. Examples of pathogens controlled are *E. amylovora* (Wodzinski *et al.,* 1987; Vanneste *et al.,* 1992; Epton *et al.,* 1994), *P. syringae* (Lindow *et al*., 1983), *Fusarium culmorum* (Kempf *et al.,* 1993), *Sclerotinia sclerotiorum* (Yuen *et al.,*

1994), *Penicillium expansum* (Montesinos *et al*., 2001), *Monilinia laxa* and *Rhizopus* spp. (Bonaterra *et al*., 2003).

2.3.1. *Pseudomonas fluorescens*

P. fluorescens is straight or curved Gram-negative rod, 0.5 to 1.0 µm in diameter and 1.5 to 5.0 µm in length, motile by polar flagella from *Pseudomonadaceae* family. Strictly aerobic, having a type of respiratory metabolism with oxygen as the terminal electron acceptor; in some cases nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically. *P. fluorescens* is positive for catalase, oxidase and arginine dehydrolase. It produces diffusible fluorescent pigments under iron limitation and can grow on a wide range of temperatures and substrates (Krieg and Holt, 1984).

Since its creation by Migula (1894), the genus *Pseudomonas* has comprised a taxon of metabolically versatile organisms that are ubiquitous in soil and water and play an important role as plant, animal, and human pathogens (Palleroni, 1992). This resulted from the ambiguous definition of the genus *Pseudomonas* as polarly flagellated strictly aerobic rods with a type of respiratory metabolism in which oxygen is used (Kwon *et al*., 2003). The heterogeneity of the genus *Pseudomonas* was significantly resolved by extensive taxonomic studies based on phenotypic (Stanier *et al*., 1966; Sneath *et al*., 1982) and genotypic techniques (Palleroni *et al*., 1973; Champion *et al*., 1980; Moore *et al*., 1996; Anzai *et al*., 2000). In particular, analysis of 16S rRNA sequences contributed to the elucidation of the natural relationships of species of the genus *Pseudomonas* at the intrageneric level and led to the significant redefinition of the genus *Pseudomonas sensu stricto*. From these studies *P. aeruginosa*, *P. fluorescens*, *P. putida* and *P. syringae* were recognized as members of the genus *Pseudomonas sensu stricto*.

Species *P. fluorescens* often has been related to the synthesis of antimicrobial compounds, like phenazines, pyoluteorin or phoroglucinol which have been described as the responsible of the control of several roots diseases (Keel *et al.,* 1989; Voisard *et al.,* 1989; Maurhofer *et al.,* 1992). However, other mechanisms of control have been described in the biocontrol of several pathogens by *P. fluorescens*, like the competence for nutrients and space described in the biocontrol of *E. amylovora* by *P. fluorescens* A506 (Wilson and Lindow, 1993). The implication of siderophores has also been described in the inhibition of *Pythium ultimum* (Loper, 1988). Finally, the capacity of induce defense responses in the host by *P. fluorescens* has been described in control of pathogens like *F. oxysporum* o *Colletotrichum orbiculare* (van Peer *et al.,* 1991; Wei *et al.,* 1996).

2.3.2. *Erwinia herbicola*

E. herbicola is a facultative anaerobic Gram-negative rod of the *Enterobacteriaceae* family. Cells are straight rods, 0.6 to 1.0 µm wide per 1.2 to 3.0 µm long, motile by peritrichous flagella. The optimum temperature of growth is 30 ºC. It has the capacity to produce yellow pigments when growth in different culture media (Brenner, 1984).

E. herbicola belongs to a diverse group of bacteria found in a variety of environments, and also include opportunistic pathogens of humans and animals also called *Enterobacter agglomerans*, *Erwinia uredovora* and *Erwinia stewartii*. Ewing and Fife (1972) proposed that the strains from clinical sources be designated as *Enterobacter agglomerans* because the characteristics of the organisms were in conformity with the genus *Enterobacter*. Strains of interest to phytopathologists have been place in the genus *Erwinia*. Currently, it is difficult, if not impossible, to distinguish strains from different sources due to the diversity in this group of organisms. Recently, based on total DNA homology and electrophoretic protein pattern similarities, strains of *Erwinia herbicola* and *Enterobacter agglomerans* have been proposed to form a new genus called *Pantoea agglomerans* (Gavini *et al.,* 1989). Across the present work the names *E. herbicola*, *E. agglomerans* and *P. agglomerans* will be used as equivalent terms.

Several are the mechanisms developed by *E. herbicola* involved in the antagonistic activity, but the most frequently described are the synthesis of antibiotic compounds like herbicolins, bacteriocins and others (Ishimaru *et al*., 1988), and the competence for nutrients and space (Hatting *et al.,* 1986). Moreover it has also been described the capacity to induce the defense responses in the host to different pathogens (Epton *et al.,* 1994).

2.4. Isolation and screening of potential biocontrol agents

The main problem of implementing biocontrol of plant diseases is due to the difficulty to obtain antagonists that suppress disease under natural conditions. Therefore, selection methods for potential biocontrol agents must be developed involving rapid and simple assays to allow the prospection of a high number of isolates. Although, before the adoption of a screening method, different aspects should be taken into account like the target disease (foliar or root diseases) or the type of pathogen implicated (fungi, bacteria, viruses).

The most common approach to select bacterial antagonists of a pathogen is screening randomly-isolated strains for biological control activity. This approach has the advantage that some strains that do not express an expected trait would not be overlooked. Nevertheless, this method involves the evaluation of hundreds or thousands of isolates to find natural antagonistic microorganisms since the efficiency of the selection process and frequency are relatively low (Howell and Stipanovic, 1980; Cook and Baker, 1983; Montesinos, 2003).

The build-up of a collection of isolates is an important step because it will determine the success of the biological control program developed. Therefore, several premises like the pathogenesis and the site where the disease will develop must take into account before confection of an isolates collection (Kinhel and Lindow, 1993).

The adoption of a suitable method to select antagonists must take into account the interaction between the host, the pathogen and the antagonist because all components are involved in the efficacy of disease reduction (Andrews, 1985). Prospection methods must accomplish two basic requirements. They have to be rapid and simple to be able to test a

great number of isolates, allow reproducing as much as possible the natural conditions where the host, pathogen and antagonist interact. In addition, designing a screening strategy is further complicated by the limited knowledge of the phenotypic features that determine success as a biocontrol agent because involves many properties in the antagonist.

Antagonists have been selected in some screening programs on basis of their capacity to inhibit the pathogen *in vitro* by means of the synthesis of some antimicrobial compounds. *In vitro* assays are cheap, easily standardized and allow mass screening of candidates. Although, most of the antagonists selected on basis of antibiosis are not capable to suppress disease under natural conditions. Possibly because they can not colonize plant surface or there are not the proper conditions to produce the antimicrobial compound which is the base of their control. For this reason, the strategy has been modified and is based on approaches where the control mechanism is not presumed, taking into account the interaction of host, pathogen and antagonist (Howell and Stipanovic, 1980; Cook and Baker, 1983).

An alternative to *in vitro* test is the screening *in planta*. Although field assays are realistic, they are expensive and require long time which makes impossible to go over a great number of isolates. An alternative to field assays is performing biosassays on sensible plant organs. These bioassays are called *ex vivo* and can be realized on different plant materials like leaves, immature fruits or flowers and permit the screening of a higher number of candidates than whole plant tests (Beer and Rundle, 1983; Handelsman *et al.,* 1990).

A disadvantage of *ex vivo* experiments is that the assay conditions must be previously determined, like the optimal pathogen and antagonist concentrations, proper inoculation method and appropriate target pathogen strain. These conditions are determined by testing dose-response relationships on disease severity on basis of the inoculation method, and virulence of pathogen strains. Besides, strains of pathogen and source of host material should be carefully considered in the chosen assays, because physiological changes associated may affect results. Moreover, plant material should be physiologically homogeneous to avoid pathogen susceptibility variations. Clonal propagation of plant material has the advantage of genetic homogeneity, which reduces statistical variation in assays. Because pathogen strains may differ in their susceptibility to an antagonist and in their virulence in the host, several strains of a pathogen should be routinely tested (Cullen and Andrews, 1984).

The best protocol is the combination of a dual screening pathway consisting of a *in vitro* stage to determine the potentiality to produce antagonism, and a screening *in vivo* under controlled environment conditions to prevent that promising antagonists not exhibiting *in vitro* antagonism be discarded. The protocol recommended by Andrews (1992) for the identification of potential biocontrol agents is shown in *Figure 1.7.*

The best candidates are sooner or later evaluated for their efficacy on plant. This evaluation is first performed under controlled environment conditions and subsequently under uncontrolled conditions. Field assays are the most determinant step in the biocontrol

development, and the results in this stage will determine the potentiality of candidates to be exploited as biocontrol agents.

In addition, potential biocontrol agents must be identified and characterized at different levels to avoid the selection of deleterious strains, to determine preliminarily the mode of action and the basic requirements for growth. The knowledge of these characteristics can help in the improvement of the efficacy and consistency of control, as well as in the production, formulation and delivery with the finality to produce the biocontrol agent at large-scale for commercially distribution.

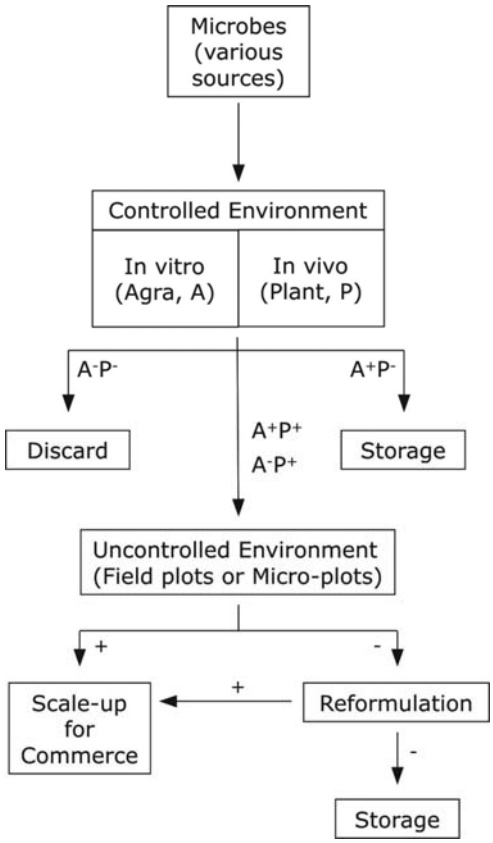

Figure 1.7. Proposed stages and sequence of assays in screening biocontrol activity. + designates activity (antagonism or disease suppression); - designates no evidence of activity (from Andrews, 1992).

Actually, the use of molecular techniques like transposon mutagenesis, make possible to determine which genes have importance in the biocontrol activity. The knowledge of these genes may be useful for the selective identification of potential biocontrol agents by means of genetic markers. Nowadays, specific markers for antibiotic genes already exist, and it is possible the detection of these genes by PCR to identify bacteria with the capacity to synthesize certain antibiotics. An example is that some strains of the species *P. fluorescens* for which specific PCR primers already exist for detection of antibiotics like phenazine-1-carboxylic acid (PCA), 2,4-diacetylphloroglucinol (Phl) (Raaijmakers *et al.,* 1997), and pyrrolnitrin (Prn) (Badosa, 2001). By means of this methodology a guided selection of

potential biocontrol agents is possible on basis of the presence of specific markers for biocontrol.

In the case of biocontrol of fire blight, most studies designed to screen putative antagonists and to investigate the mechanisms of biological control have employed culture media and immature pear fruits to measure inhibition of *E. amylovora* by antagonistic strains (Wrather *et al*., 1973; Beer and Rundle, 1983; Isenbeck and Schultz, 1985; Ishimaru *et al*., 1988; Nicholson *et al*., 1990; Vanneste *et al*., 1992; Kearns and Mahanty, 1993). However, the research of biological control of fire blight is now focused on the interaction of an antagonist, usually bacterial, and *E. amylovora* on stigmatic and hypanthial surfaces of pear and apple blossoms (Vanneste, 1996; Pusey, 1998). In this system the selection of antagonists is independent of the mode of action developed by the antagonists. For this reason, the suppression of pathogen populations on blossoms has been defined as an advanced approach to identify effective antagonistic strains (Andrews, 1985; Mercier and Lindow, 1996; Pusey, 1997).

2.5. Improvement of biocontrol agents functionality

The main problem of biocontrol is the limited efficacy when the biocontrol agent when is applied under field conditions. This problem can be solved by means of improving the ability of biocontrol agents to colonize and survive in the environment, and favor conditions expressing the biocontrol mechanism. Therefore, it is necessary to understand the mechanisms involved in the interaction between the pathogen and the biocontrol agent and the factors that influence growth of both microorganisms in the natural environment to develop techniques that favor the antagonistic activity when the antagonist is introduced in a concrete environment.

The efficient establishment and colonization of antagonists on plant surfaces will require proper inoculum production methods and formulations. The most direct way to obtain an improvement of the colonization and survival is by means of amendments to the biocontrol agent, incorporating nutritional additives that are often limitant in the ambient, adherents to improve the adhesion and increase the time of residence, or UV light protectants to improve survival. Adjuvants or amendments typically used consisted of surfactants, suspending agents, humectants, stickers, buffering agents, microbial nutrients, selective inhibitors, salts, and other substances to promote the effectiveness of introduced and indigenous biocontrol organisms (Sutton and Peng, 1993).

The survival also can be improved modifying the adaptation of the biocontrol agent to the conditions of hydric stress by means of osmoadaptation based on the incorporation into cell cytoplasm of osmoprotectants like betaine or trehalose (Csonka and Hanson, 1991; Miller and Wood, 1996).

Another strategy to improve the effectiveness of the biological control is the combination of several strains or species of biocontrol agents to obtain synergic or complementary effects.

These mixtures improve the colonization of the ambient, survival due to the benefits of a mixed population which have better adaptability to environmental variations, and the efficacy because there are many mechanisms involved in pathogen control and reducing the probability to select resistant strains of the pathogen to the biocontrol agent. Besides, the development of mixtures of antagonists permits to increase the range of action against pathogens and in different hosts (Janisiewicz, 1996).

Finally, genetically modified organisms (GMOs), obtained by non-recombinant techniques can be used to improve the efficacy. Biotechnology can be used to manipulate genes to increase efficacy by enhancing colonizing ability or by potentiation of the production of several compounds (antibiotic or siderophore synthesis). The novel strains can acquire new characteristics such as the ability to produce several antibiotics, toxins, EPS even from other microorganisms which confers to them antagonistic potential and enhanced and more stable activity. However, the major limitation is the low number of known genes involved in biocontrol as well as in the colonization and antagonistic activity. Besides, GMOs can only be used in confined assays and its use is not allowed in commercial orchards.

2.6. Production, formulation and delivery of biocontrol agents

The last step in the development of biological control agents for commercial distribution is scale-up and industrial production. In many cases there are difficulties in the production by liquid fermentation at high biomass and in achieving repetitiveness in quality, as well as problems in formulation and preservation of the biocontrol agent. The preparation of biocontrol agents to use as biopesticides in commercial orchards include large-scale production of the microorganism maintaining its antagonistic capacity, formulation for optimizing its efficiency, dehydration of the product to improve the preservation and applicability and the methodology of delivery. Besides, the commercialization of biocontrol agent needs to overcome toxicity tests and registration procedures.

The production process has to optimize the increase of viable biomass, which must also be of the desired form, consistent in quality, and competent with respect to colonizing ability. Optimization implies seeking the lowest input cost per unit of viable product produced. Therefore, cheap substrates, efficient conversion (specific growth yield), and fast growth are among the key considerations (Andrews, 1992).

A formulated product with agricultural application should possess several desirable characteristics including adequate market potential, easy preparation and the application, stability during transportation and storage, abundant viable propagules and good shelf-life, sustained efficacy, and acceptable cost (Andrews, 1992; Lumsden *et al*., 1995).

During formulation processes care must be taken to avoid drastic decrease of biocontrol agent viability. End products from fermentations can be formulated as dusts, wettable powders, granules, pellets, gels, or emulsifiable liquids (*Figure 1.15*). Dusts consist of the antagonist dried to a carrier. Formulations based on such materials as clays, peat,

vermiculite, or bran are being supplemented or replaced by encapsulation of the antagonist within cross-linked matrix organic polymers, which improve consistency, retention, and protection of the biocontrol agent (Andrews, 1992).

Figure 1.8. Available biological products on different formulates.

Moreover, to be effective, biocontrol organisms depend on methods and strategies used to introduce and maintain the organisms in the crop (Sutton and Peng, 1993). An ideal biocontrol strategy introduces the antagonists only when and where they are needed or are most effective, and minimizes wasteful application of inoculum to nontargets.

Inoculative or classical biocontrol involves the release of a relatively small level of biological control agent in an area in which they are not already present, with intent to establish a permanent population. This strategy involves a one-time or occasional inoculative release (Mathre *et al*., 1999). Augmentative biocontrol involves releasing of moderate amounts of a biological control agent complemented with amendements or modification of the environment in order to favor its growth. In this case, the biocontrol agent is applied with the intent that it will spread and protect the plant at one or more distant places. Plant growth promoting rhizobacteria (PGPR) falls into this caterogy (Kloepper *et al*., 1980). While, inundative biocontrol involves the release of large amounts of a biological control agent relative to the numbers of a target species, in expectation of a rapid effect. There is no implication that the released biological control agent will establish a permanent population, because they are applied when and where needed. Nearly all microorganisms commercialized for biological control of plant disease fall into this group (Mathre *et al*., 1999) and mimics chemical pesticides.

2.7. Implementation of biocontrol

Despite intensive research, only few effective biocontrol products have been commercialized. This is due to the fact that first steps of biocontrol strategy development are assumable for research groups. However, the necessity of the development of field, toxicological and

formulation studies as well as the practical problems in industrial production, application or commercialization, limit the development of commercial biocontrol products. Thus, most studies on biocontrol are incomplete to get a commercial product. Besides, completing data require many years of development and industries are not attracted for products which may have reduced range of application and can be difficult to be registered under current rules, especially in the European Union.

Another cause is the fact that the market for chemical pesticides is large and lucrative, whereas the market for biopesticides is actually much reduced and suppose a risk that many manufactures are not disposed to assume. In *Table 1.8* there are some advantages and disadvantages of chemical and biological pesticides as shown by Andrews (1992).

Table 1.8. Advantages and disadvantages of chemical and biological pesticides (From Andrews, 1992)

	Chemical	**Biological**
Product development		
R + D costs per product	20 M €	0.8-1.6 M €
Toxicology testing	10 M €	0.5 M €
Patentability	Straightforward	Risky
Discovery	Undirected screens: assess 20.000 candidates per new product	Directed screens for specific targets: success rate?
Market size required for profit	40 M € per year	5-20 M €: 1.6 M € may be profitable
Product use		
Efficacy	High: 98-100%	Moderate, often < 50%
Spectrum	Broad	Narrow
Activity	Prophylactic; therapeutic	Prophylactic
Speed of action	Typically fast	Typically slow
Human healthy/ environment effects	Well-established adverse effects	information lacking

3. Biological control of fire blight

The need to develop and implement biocontrol of fire blight has resulted from several factors, including the increasing importance of the disease, the moderate efficacies of existing control measures, development of resistance in *E. amylovora* to the antibiotic streptomycin and social demand to enhance the safety and sustainability of agricultural production systems. Besides *E. amylovora* continues to spread to new countries, many of which do not allow the use of antibiotics for disease control.

Over the last 20 years, biological control of fire blight has advanced from basic research to a component of integrated disease management programs. This advance has been propelled by selection of effective antagonist strains, by enhanced knowledge of the mechanisms by which these strains suppress disease and by increased understanding of the ecology of bacterial epiphytes on plant surfaces. Moreover, orchardists of some countries have begun to

accept biological control as a complementary strategy, which can be used effectively with other forms of disease suppression (Johnson and Stockwell, 2000).

Flowers are the main targets for natural infections of host plants. *E. amylovora* colonizes the stigma of flowers before infecting through the nectaries (Thomson, 1986). Surface of the stigma is the site where biological control agents must interact with and successfully antagonize *E. amylovora*. When the blossoms opens, the stigma is generally devoid of bacteria, but with time, the exposed stigmas are colonized and high populations of bacterial epiphytes, including *E. amylovora*, develop on the surfaces (Thompson, 1986).

Pre-emptive colonization of flowers by bacterial antagonists has shown to prevent increases in population size of *E. amylovora* and subsequent infection of the blossom (Wilson *et al*., 1992; Johnson *et al*. 1993; Wilson and Lindow, 1993; Johnson and Stockwell, 1998). Biological control of fire blight is produced when a bacterial antagonist establishes and develops a large population on the stigmatic surface prior to the establishment of *E. amylovora* (Johnson and Stockwell , 2000).

Biological control of the blossom blight phase of fire blight has been demonstrated as an alternative to antibiotics (Beer *et al*., 1984; Vanneste, 1996; Pusey, 1997; Bogs *et al*., 1998), or a measure that could complement with the use of antibiotics (Lindow *et al*., 1996, Stockwell *et al*., 1996; Pusey, 1997). Besides, suppression of floral infections reduces the inoculum of *E. amylovora* available for other phases and cycles of the disease, including shoot infections during the same season and floral infections in the following season (Stockwell *et al*., 1996; Johnson and Stockwell, 2000).

Nowadays, control of fire blight has been achieved by spraying suspensions of the antagonistic strain onto apple (Beer *et al*., 1984; Vanneste *et al*., 1992) and pear (Wilson and Lindow, 1993; Mercier and Lindow, 1996; Nuclo *et al*., 1998) flowers before inoculating with *E. amylovora*. For this reason, research on the biological control of fire blight has been focused on the interaction between an antagonist, usually bacterial, and *E. amylovora* on stigmatic and hypanthial surfaces of pear or apple blossoms.

The earliest reports on biological control of fire blight have been reviewed by van der Zwet and Keil (1979) beginning in the early 1930s when bacterial strains antagonistic to *E. amylovora* were isolated and shown to have a tendency to reduce the percentage of fire blight infection when applied to blossom. Subsequently many workers have investigated both Gram-positive and Gram-negative bacteria for their antagonistic properties towards *E. amylovora*, especially *Pseudomonas* spp. (Lindow, 1984) and *E. herbicola* (Beer and Rundle, 1983).

E. herbicola and *P. fluorescens* remain the most frequently studied potential biological control agents of fire blight, and *P. fluorescens* A506 and *E. herbicola* C9-1 are already registered as biological products for the fire blight control. Capacity of naturally occurring microbial antagonists from these species have the ability to produce antibiotic compounds *in vitro* against *E. amylovora* (Vanneste *et al*., 1992; Wodzinski *et al*., 1996; Wright *et al*., 2001;

Stockwell *et al*., 2002), to inhibit infections caused by *E. amylovora* in immature pear fruits (Beer and Rundle, 1983; Vanneste *et al*., 1992) and also control infections in pear and apple trees (Kearns and Hale, 1993) and in ornamental plants (Wilson *et al*., 1992).

These biocontrol agents have been isolated from shoots, leaves, or blossoms of apple or pear trees and identified by their ability to inhibit *E. amylovora* in immature pear fruits (Beer and Rundle, 1983; Vanneste *et al.,* 1992) or in pear blossoms (Pusey, 1997). Although, one of the few registered products for biocontrol of fire blight is based on an isolated from *P. fluorescens* species, concretely, *P. fluorescens* strain A506 (Lindow, 1984).

P. fluorescens A506 was isolated from pear in California (Lindow, 1984), and has shown the capacity to decrease the incidence of fire blight as well as to suppress the severity of frost injury caused by ice nucleation active strains of *P. syringae* (Lindow *et al.,* 1996). Strain A506 is a good colonizer of pear and apple stigmas. On average, a 40 to 60 % of reduction in incidence of fire blight on blossoms has been described along several plot assays in different localizations (Johnson *et al.,* 1993; Lindow *et al.,* 1996).

A second antagonist, *E. herbicola* C9-1, was isolated from apple in Michigan (Ishimaru *et al.,* 1988) and has shown an effective control of fire blight in plot assays (Johnson *et al*., 1993; Stockwell *et al.,* 1996). Like A506, C9-1 is a good colonizer of stigmatic surfaces of pear and apple. Treatments based on C9-1 have decreased the incidence of fire blight between 50 and 80% (Johnson *et al.,* 1993).

These antagonists have been the precedents in the biocontrol of fire blight. Nowadays, several other strains, mainly of *E. herbicola* species, have shown promise for suppression of blossom infection by *E. amylovora*. These strains include *Eh*I9n13 (Wilson *et al.,* 1990), *Eh*252 (Vanneste *et al.,* 1992), *Eh*112Y (Wodzinski *et al.,* 1994), *Eh*318 (Wright and Beer 1996), *Eh*1087 (Kearns and Hale, 1996), and *Eh*325 (Pusey, 1997) (*Table 1.9*).

Table 1.9. Efficacy of different biological control agents of fire blight obtained against blossom blight in comparison to streptomycin.

Active ingredient	**Product name**	**Host**	**Effectiveness (% control)**	**References**
E. herbicola C9-1	BlightBan C9-1	Pear	50-80	Ishimaru *et al.* (1988)
P. fluorescens A506	BlightBan A506	Pear	40-60	Lindow *et al. (*1996)
E. herbicola Eh252	n.r.	Pear	55	Stockwell *et al.* (2002)
E. herbicola Eh1087	n.r.	Apple	70-80	Kearns and Hale (1996)
E. herbicola E325	n.r.	Apple	73-79	Pusey (2002)
Streptomycin	Agrimycin 17	Apple	90	Jones and Byrde (1987)

n.r. not registered.

The mechanisms involved in biological control of fire blight depend on the strain. Production of antibiotics and site and nutrient competition are considered to be the main mechanisms used by *E. herbicola* and *P. fluorescens* to antagonize *E. amylovora* (Wilson and Lindow, 1993; Vanneste, 1996).

In the case of *E. herbicola*, it has been suggested that antibiotics are involved in the inhibition *in planta*, because production of antibiotics is a common phenotype of *E. herbicola* in synthetic culture media (Wodzinski *et al.,* 1994). These antibiotics show common characteristics and are referred to as herbicolins (Ishimaru *et al.,* 1988), microcins (Vanneste *et al.,* 1998) or pantocins (Wright *et al.,* 2001). It has been observed that some strains of *E. herbicola* produce a single antibiotic (strains *Eh*252, *Eh*112Y and *Eh*1087) (Beer *et al.,* 1984; Vanneste *et al.,* 1992; Kearns and Hale, 1996), whereas others produce multiple compounds (strains *Eh*318, and *Eh*C9-1) (Ishimaru *et al.,* 1988; Wright and Beer, 1996).

Thus, although, the importance of antibiotic production by *E. herbicola* strains has not been evaluated directly, correlative evidence indicates that herbicolines production contributes to the effectiveness strains. Herbicolines decreased the development of fire blight symptoms on immature pear fruit when fruits are inoculated by sensitive strains to both antibiotics, while only partially reduced the severity of symptoms when a herbicolin-insensitive mutant of *E. amylovora* was inoculated (Ishimaru *et al.,* 1988).

The implication of the antibiosis in the inhibition of *E. amylovora* has also been demonstrated in strain *Eh*252. A Tn5-mutant of *Eh*252 that no longer produces its antibiotic did not reduce symptom severity on immature pear fruits as well as its wild-type parent (Vanneste *et al.,* 1992). However, the non-antibiotic-producing mutants of *Eh*252 still retained some ability to suppress fire blight development indicating that factors other than antibiotic production are involved in biocontrol of fire blight. It has also been reported that Tn5-induced mutants of *E. herbicola Eh*112Y that have lost the ability to produce antibiotic were as effective as the wild-type strain in protecting immature pear fruits from fire blight (Beer *et al.,* 1984). In this case, the implication of other mechanisms of control involved in disease suppression is clear.

The partial suppression of disease under conditions of mutated antibiotic activity is an indication that site and nutrient competition as well as other mechanisms also contribute to the overall effectiveness of these strains. The control of fire blight can not be attributed strictly to one mechanism, and the most probable situation is the involvement of several mechanisms with a synergic effect in the inhibition of *E. amylovora*.

The relative effectiveness of most of antagonists is apparently correlated with the antibiotic production. However, antibiotic production has not been demonstrated for some strains, and other inhibition mechanisms seem to be involved. In contrast of most of *E. herbicola* antagonists, antibiotics inhibitory to the growth of *E. amylovora* have not been detected in cultures of *P. fluorescens* strain A506 (Wilson and Lindow, 1993). This bacterium apparently suppresses the pathogen by competing for sites and nutrient required for the growth of *E. amylovora*. Concretely, the pre-emptive sequestration of mutually required growth-limiting resources is the probable cause of pathogen suppression (Wilson and Lindow, 1993).

CONTEXT OF THIS WORK

The actual trend of crop protection has been reoriented to a rational use of pesticides and to a reduction of the number of registered active ingredients to those certainly unavoidable, more selective, less toxic and with a lower negative environmental impact (Gullino and Kuijpers, 1994; Ragsdale and Sisler, 1994). Besides, in the case of fire blight, the low effectiveness of the currently available methods for disease control, the development of pathogen resistance, the banned use of some active products in Europe (e.g. streptomycin) and the widespread of fire blight across different areas of Spain, make necessary the development of alternative control methods. For this purpose, it was proposed the development of a biological control agent obtained from our specific climatic conditions for the preventive treatment of fire blight and for preventing dispersion of existing focus and introduction of fire blight in protected fruit free production areas of Spain.

This study has been developed under one research project of the “Programa Nacional de Ciencias Agrarias”, “Estudios epidemiológicos y evaluación de estrategias de control integrado de fuego bacteriano (*Erwinia amylovora*) en España” (CICYT AGF98-0402-C03-01), and another of the “Programa Nacional de Recursos y Tecnologías Agroalimentarias”, “Optimización de los métodos de detección, prevención y control de fuego bacteriano de las rosáceas en España” (CICYT AGL-2001-2349-C03-01), both granted by the “Comisión Interministerial de Ciencia y Tecnología” of the Spanish Government.

This work is framed in the research lines of the Plant Pathology group of the “Universitat de Girona”. The research priority are focused in developing new strategies of disease control in order to minimize the use of chemical pesticides in fruit tree management programs.

OBJECTIVES

The main objectives of this thesis were:

1. To characterize a collection of strains of *E. amylovora* isolated from Spain and optimize *ex vivo* and *in planta* model systems for interaction studies.

2. To isolate and constitute a collection of biocontrol agents of fire blight, mainly composed of *E. herbicola* and *P. fluorescens* strains.

3. To characterize a selection of the best biological control agents and determine the putative mechanisms of action involved in fire blight control.

CHAPTER 2

Characterization of strains of Erwinia amylovora *and optimization of model systems for interaction studies*

INTRODUCTION

Appropriate management of fire blight caused by *E. amylovora* is an important challenge due to fire blight is an economically important disease that affects apple and pear production worldwide and spreads through new growing regions. For this reason, *E. amylovora* has been extensively studied at different levels like morphological characteristics, physiology, nutrition or serology with the purpose to understand the pathogenesis, the diversity and the ecology of the bacterium, to know fire blight epidemiology and to improve and develop control and detection methods.

General characterization and identification based on cultural and morphological characteristics, physiology, nutrition, serology and other traits provide clear and reliable placement of bacteria into groups as well as provide a means to separate *E. amylovora* this species from other groups of bacteria (Paulin, 2000). Besides, strain identification is necessary for understanding the dispersal and spread of the pathogen.

Most of characterization studies compare virulent and non-virulent strains, in order to provide a better understanding of the pathogenesis and the ecology of this bacterium. Nevertheless, differences in physiology, serology and other characters between non-virulent and virulent forms of the pathogen have not been found with the exception of the presence of exopolysaccharide that has been found in virulent strains (Belleman and Geider, 1992). Therefore, in general relations between phenotypic characteristics and pathogenesis have not been observed. Although, development of molecular techniques have provided a tool to determine the involvement of several genes in pathogenesis, like *hrp* and disease-specific genes (*dsp*) (Belleman and Geider, 1992; Tharaud *et al*., 1994).

In relation to the host range, there are not described pathovars from among approximately 200 plant species in 40 rosaceous genera studied where fire blight induces symptoms. Although, host species specificity has been observed mainly associated to strains isolated from ornamental plants (Beer *et al*., 1996) as well as a differential virulence on apple (Norelli *et al*., 1984) and pear. In spite of this differential virulence described, there are not exhaustive studies of characterization of virulence among a wide collection of *E. amylovora* strains.

When the species diversity has been studied on basis of general characteristics a great homogeneity was observed. Such homogeneity within the *E. amylovora* species has been recognized for a long time and confirmed when strains isolated from different hosts and regions have been studied concerning cultural, biochemical, and physiological characteristics (Vantomme *et al*. 1982, Gavrilovic and Arsenijevic 1999). On the basis of these studies, no characteristics have been found that could distinguish strains from different geographical origins, host plants or isolated at different years.

However, recent results using molecular techniques (RAPD, pEA29-PCR, genes RFLPs, rep-PCR fingerprinting, PCR ribotype, PFGE) have shown differences between strains of diverse origins (geographical, host plant) (Mcmanus and Jones, 1995; Bereswill *et al*., 1997; Kwon *et al*., 1997; Zhang and Geider, 1997; Manulis *et al*., 1998; Kim and Geider, 1999; Jock *et al*., 2000; Rico *et al*., 2004). Even so, only three major groups of strains have been described: Maloideae, *Rubus* and “Hokkaido” (Momol *et al*., 1997). The main criteria that distinguish these three groups are their specialization toward host and the molecular fingerprinting analyses. In addition, there are subgroups within each group that have been discriminated by molecular and biochemical techniques, like PFGE patterns that relate strains in function of their geographical origin. Such, genetic diversity can be used as a tool to identify possible sources of infection, to assist in gene mapping, to aid in individual strain identification, to study the population genetics of species and to serve as characters in molecular phylogenetic studies (Bowditch *et al*., 1993).

One of the reasons of the limited success of disease management is the inadequate understanding of the host range and diversity of *E. amylovora* strains. The pathogen diversity and host range characterization of *E. amylovora* is important to establish a plant quarantine strategy, to select resistant cultivars in breeding and genetic engineering programs, to trace the pathogen spread and to identify possible sources of infection. Also, a properly designed screening process of biocontrol agents should be performed using representative strains of the pathogen.

OBJECTIVES

This chapter was focused on the selection of virulent representative strains of *E. amylovora* or at least of the Spanish strains, to be used in the selection of potential biocontrol agents of fire blight. The specific objectives were to:

- constitute a collection of new *E. amylovora* strains from Spanish commercial orchards and nurseries, to be included in a more extensive collection of representative strains from different sources.
- study the diversity of the strains collection attending to cultural, nutritional, biochemical, serological, molecular and virulence levels in order to select representative strains to perform interaction studies with potential biocontrol agents.
- optimize pathosystem models based on blossom and shoots to be applied on the selection of a potential biocontrol agent of fire blight and for interaction studies.

MATERIALS AND METHODS

In this chapter, a collection of *E. amylovora* strains from different origins (plant host, location are characterized according to cultural, nutritional, biochemical, serological, molecular and virulence studies.

1. Isolation of new *E. amylovora* strains

Strains were isolated from 20 symptomatic samples collected from apple and pear trees and from ornamental plants, proceeding from different commercial orchards and nurseries localized in Catalunya. Isolates were obtained from samples which gave signals using enrichment DASI-ELISA (Gorris *et al.*, 1996) and PCR with primers described for plasmid pEA29 (Bereswill *et al.*, 1992).

1.1. Sample processing and extraction

Selected parts of plant material from each sample were disinfected with sodium hypochlorite (1%) and washed with sterile water, cut and macerated in 1 mL of antioxidant maceration buffer (*Appendice 1*). The extracts were spread in 0.1 mL aliquots onto plates of King's B medium (KB) and 5% sucrose nutrient agar medium (SNA) (*Appendice 2*), and incubated at 25 ºC. Colonies were scraped from the plates and suspended in 1 mL of sterile distilled water. At the same time, the extracts were enriched in CCT and KB medium (*Appendice 2*). The enrichment was performed by mixing 0.6 mL of extract with an equal volume of enrichment medium, and incubation at 25 ºC for 72 without shaking. Bacterial suspensions and enrichments were boiled at 100 ºC during 10 min in a thermoblock (Multibloc, Selecta, Spain) and maintained at –80 ºC until use.

1.2. DASI-ELISA analysis

The analysis by enrichment DASI-ELISA was performed using the technique described by Gorris *et al.* (1996) with a commercial kit (Plant Print Diagnostics S. L., Valencia, Spain). ELISA plates were filled with 200 µL of a dilution 1:100 of rabbit anti-*E. amylovora* polyclonal immunoglobulins in carbonate buffer, pH 9.6 (*Annex 1*). The plate was incubated at 37 ºC for 4h, and washed three times with physiological buffered water (AFT) (*Annex 1*). Then, 200 µL of boiled extracts were added to each well. A suspension of the non virulent mutant *E. amylovora* PMV6076 was added to some wells as positive control while the negative control was done by addition of extraction buffer. Plates were incubated at 4 ºC for 16h, and washed as above. Then, 200 µL of a dilution 1:1000 of specific *E. amylovora* mouse monoclonal antibodies in AFT plus 0.5% bovine serum albumin (BSA) were added. The plates were incubated at 37 ºC for 2 h and washed three times with AFT-plus 1% tween20. Subsequently, 200 µL of a dilution 1:1000 of goat anti-mouse immunoglobulins conjugated with alkaline phosphatase in AFT were added. The plate was incubated at 37 ºC for 2 h. The last wash was performed as above before the addition of 200 µL of a solution of 1mg·mL^{-1} of p-nitrophenylphosphate in substrate buffer (*Annex 1*). Finally, the plate was incubated at room temperature and the optical density (OD) was read at 405 nm after 30, 60 and 90 min with a multiscan photometer (Labsystems MultisKan RC, Helsinki, Finland) (*Figure 2.1*) using the ASCENT software (Ascent Research Edition version 2.1, Labsystems, Helsinki, Finnland). The ELISA test was considered negative when the OD reading of sample wells was lower than twice OD of that in the negative control. Whereas, it was considered positive when was greater than twice OD in the negative control.

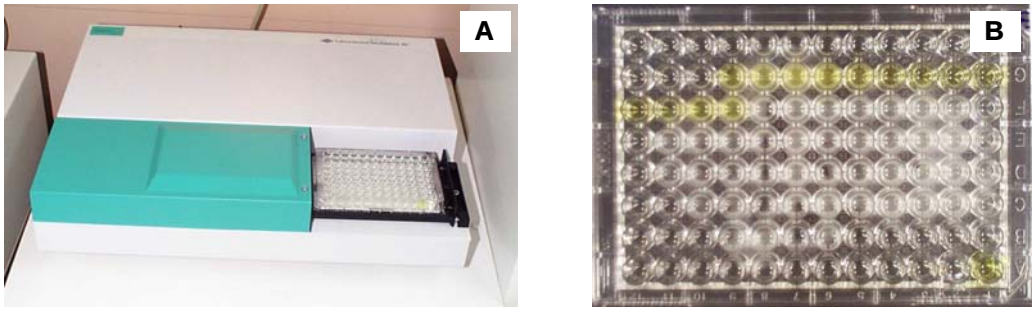

Figure 2.1. Optical density multiscan (**A**) and ELISA plate revealed with alkaline phosphatase substrate (**B**).

1.3. PCR analysis

PCR was performed using the primers described for plasmid pEA29. Bacterial DNA extraction from sample extracts was performed following the isopropanol extraction procedure (Llop *et al.*, 1999). The total DNA was prepared from bacterial suspensions (1 mL) or from enriched extracts (600 µL). The suspensions were centrifuged at 13.000 rpm for 5 min. The supernatant was discarded and the pellet was re-suspended in 500 µL of DNA extraction

buffer (*Annex 1*) and incubated under shaking with a vortex for 1 h. The suspensions were centrifuged at 5.000 rpm for 5 min. Then, 450 µL of supernatant were transferred to a fresh Eppendorf tube and mixed by inversion several times with an equal volume of isopropanol. The tubes were incubated at room temperature for 1 h. The suspensions were centrifuged at 13.000 rpm for 10 min, the supernatant was discarded and the pellet dried for about 1 h at room temperature by standing tubes upside down on a tubes rack. The pellet was re-suspended in 200 µL of sterile Milli-Q water, allowed for complete re-suspension, and kept at –20 ºC until their use.

Standard PCR for the amplification of a specific DNA fragment of pEA29 plasmid was performed with the primers described in *Table 2.1*. PCR reaction was carried out in a volume of 50 µL, containing 10 µL of DNA extraction, 1 µL of dNTPs 10 mM (Amersham Pharmacia Biotech, Sweden), 25 pmol of each primer, 3 µL Cl_2Mg 50 mM, 5 µL PCR buffer 10X, 28.8 µL of sterile demineralised water and 0.2 µL of Taq DNA polimerase 5 $U \cdot \mu L^{-1}$ (GIBCO, Life Technologies, Barcelona, Spain) (Bereswill *et al.,* 1992). Amplifications were performed with a GeneAMP® PCR system 9700 (PE Applied Biosystems, Forster, New York, USA). The PCR program consisted of an initial denaturation at 94 ºC for 5 min followed by 40 cycles of 94 ºC for 30 s, 52 ºC for 20 s, 72 ºC for 30 s, and a final elongation at 72 ºC for 10 min.

Table 2.1. Size of the DNA amplified fragments obtained using different primers according to the annealing temperature

Target gene	**Primer**	**Sequence 5' → 3'**	**Temperature[1]** (ºC)	**Product[2]** (bp)	**Reference**
pEA29	PEAA	CGGTTTTTAACGCTGGG	45	900	Bereswill *et al.,* 1992
	PEAB	GGGCAAATACTCGGATT			

[1]Annealing temperature corresponding to each pair of primers.
[2]Length of the DNA fragment amplified for each PCR.

Samples of PCR products obtained were separated by horizontal electrophoresis (Mini-Sub® Cell GT, Bio-Rad, Barcelona, Spain) on a 1.2% agarose gel (Roche Diagnostics, Barcelona, Spain) in 1X TAE buffer (*Annex 1*) at 70 V for 45 min. The gel was stained with ethidium bromide for 30 min, and the PCR products were visualized with a UV transilluminator (TFX-20M, Vilver Lourmat, France). The expected PCR product has a size of 900 bp according to Bereswill *et al*. (1992). *E. amylovora* strain PMV6076 was included as positive control while the negative control was performed with water. Colonies that gave an amplified product of the corresponding molecular weight were considered as strains of *E. amylovora*.

1.4. Isolation of bacteria

The extracts from field samples that gave positive reaction in enrichment DASI-ELISA and PCR were spread in 0.1 mL aliquots on plates of KB medium and sucrose nutritient agar (SNA) (*Annex 2*) amended with 50 $\mu g \cdot mL^{-1}$ cycloheximide to avoid fungal growth. Plates were incubated for 48 h at 25 ºC. White non-fluorescent colonies on KB medium, producing dome shaped colony morphology growth on SNA agar plates were selected as putative *E.*

amylovora. Single colonies were streaked on LB agar to get pure cultures. Suspected isolates were newly tested by DASI-ELISA and PCR using the pEA29 primers as described above. For long-term preservation, confirmed *E. amylovora* strains were stored in 20% glycerol at –80 ºC.

2. Characterization of *E. amylovora* strains

A total of 53 *E. amylovora* strains from different origins (*Table 2.2*) were characterized by cultural, nutritional and enzymatic identification tests, PCR reaction using different primers, DASI-ELISA analysis and pathogenicity in immature pear fruits.

Table 2.2. E. amylovora strains used in this work

Strain	Host plant	Source	Country	Year of isolation
EPS100	*Pyrus malus*	E. Montesinos-P. Vilardell	Girona (Spain)	1997
EPS101	*Pyrus communis*	E. Badosa-J. Cabrefiga	Lleida (Spain)	1999
USV1000	*Pyrus communis*	F.Garcia-C.Monton	Lleida (Spain)	1999
USV1043	*Pyrus communis*	F.Garcia-C.Monton	Lleida (Spain)	1999
USV2194	*Pyrus communis*	F.Garcia-C.Monton	Lleida (Spain)	1999
USV2773	*Pyrus communis*	F.Garcia-C.Monton	Lleida (Spain)	1999
USV4300	*Pyrus communis*	F.Garcia-C.Monton	Lleida (Spain)	1999
USV4408	*Pyrus communis*	F.Garcia-C.Monton	Lleida (Spain)	1999
USV4500	*Pyrus communis*	F.Garcia-C.Monton	Lleida (Spain)	1999
USV4501	*Pyrus communis*	F.Garcia-C.Monton	Lleida (Spain)	1999
USV4512	*Pyrus communis*	F.Garcia-C.Monton	Lleida (Spain)	1999
USV4576	*Pyrus communis*	F.Garcia-C.Monton	Lleida (Spain)	1999
CUCM273	*Malus sylvestris*	S.V. Beer	USA	1971
USV4320	*Pyrus communis*	F.Garcia-C.Monton	Lleida (Spain)	1999
NCPPB311	*Pyrus communis*	M.R. McLarty	Canada	1952
NCPPB595	*Pyrus communis*	J.E. Crosse-W. Zeller	England	1958
NCPPB683	*Pyrus communis*	R.A. Lelliot	England	1959
NCPPB1734	*Pyrus communis*	M.K. Abo-el-Dahab	Egypt	1965
NCPPB1819	*Crataegus* sp.	-----	USA	1966
NCPPB2080	*Pyrus* communis	D.W. Dye	New Zealand	1968
NCPPB2791	*Pyrus communis*	M.N. Schroth	USA	1975
NCPPB3159	*Malus sylvestris*	A. Calzolari	Netherlands	1981
NCPPB3548	*Eribotrya japonica*	Y. Oktem	Turkey	1987
CFBP1430	*Crataegus* sp.	J.P. Paulin–R. Samson	France	1972
PMV6076	*Crataegus* sp.	*hrp-dsp*-deletion mutant of CFBP1430	France	1990
OMP-BO1185	*Crataegus* sp.	A. Calzolari	Italy	1994
IVIA 1614.2	*Crataegus* sp.	M.M. López	Segovia (Spain)	-----
Ea115.22	*Cydonia oblonga*	S. Bobev	Bulgary	1989
UPN500	*Pyrus communis*	D. Berra	Navarra (Spain)	1998
UPN504	*Pyrus communis*	D. Berra	Guipúzcoa (Spain)	1998
UPN506	*Pyrus malus*	D. Berra	Guipúzcoa (Spain)	1998
UPN513	*Pyrus communis*	D. Berra	Guipúzcoa (Spain)	1998
UPN514	*Pyrus communis*	D. Berra	Guipúzcoa (Spain)	1998
UPN524	*Pyrus malus*	D. Berra	Navarra (Spain)	1998

(Continue)

Strain	Host plant	Source	Country	Year of isolation
UPN529	*Pyracantha* sp.	M. Borruel	Guipúzcoa (Spain)	1997
UPN530	*Pyrus communis*	M. Borruel	Navarra (Spain)	1997
UPN536	*Pyrus malus*	D. Berra	Guipúzcoa (Spain)	1998
UPN544	*Crataegus* sp.	M. Borruel	Navarra (Spain)	1998
UPN546	*Cydonia* sp.	M. Borruel	Guipúzcoa (Spain)	1998
UPN562	*Pyracantha* sp.	M. Borruel	Navarra (Spain)	1998
UPN575	*Pyrus malus*	M. Borruel	Navarra (Spain)	1998
UPN576	*Pyracantha* sp.	M. Borruel	Navarra (Spain)	1995
UPN588	*Pyracantha* sp.	M. Borruel	Navarra (Spain)	1998
UPN609	*Sorbus sp.*	M. Borruel	Navarra (Spain)	1998
UPN610	*Pyracantha* sp.	M. Borruel	Navarra (Spain)	1998
UPN611	*Cotoneaster* sp.	M. Borruel	Navarra (Spain)	1998
EAZ1	*Pyrus communis*	Mi. Cambra	Zaragoza (Spain)	2000
EAZ4	*Pyrus communis*	Mi. Cambra	Zaragoza (Spain)	2000
EAZ7	*Pyrus communis*	Mi. Cambra	Zaragoza (Spain)	2000
EAZ9	*Pyrus communis*	Mi. Cambra	Zaragoza (Spain)	2000
EAZ13	*Pyrus communis*	Mi. Cambra	Zaragoza (Spain)	2000

EPS, Escola Politècnica Superior-Universitat de Girona (Spain); USV, Unitat del Servei de protecció dels Vegetals (Spain); CUCM, Cornell University Collection of Microorganisms, Ithaca (USA); NCPPB, National Collection of Plant Pathogenic Bacteria, Plant Pathogen Laboratory, Harpenden, Hersfordshire (England); CFBP, Collection Française de Bactèries Phytopathogènes, INRA Angers (França); PMV, Laboratoire de Pathologie Moleculaire et Végétale INRA/INA-PG, Paris (France); OMP-BO, Università degli Studi di Bologna; IVIA, Instituto Valenciano de Investigacions Agrarias, Valencia (Spain); UPN, Universidad Publica de Navarra (Spain); EAZ, Servicio de Investigaciones Agrarias de Aragón (Spain).

2.1. Identification by cultural, nutritional and enzymatic tests

Strains were identified on the basis of growth characteristics in different media and by means of the Analytical Profile Index (API) 20E (Biomerieux, France) and Biolog system (Biolog, Inc., Hayward, California, USA).

2.1.1. Growth characteristics on different media

The media used were King's B, sucrose nutrient agar, CCT (CCT), and Miller-Schroth (MS) (*Annex 2*) (*Table 2.3*).

Table 2.3. Characteristic growth of *E. amylovora* on culture media (Paulin, 2000)

Medium	Colonies appearence	Reference
KB	White circular mucoid colonies without fluorescence	Paulin and Samson (1973)
SNA	Domed circular mucoid colonies	Billing *et al*. (1961)
CCT	Smooth, large, pulvinate, light blue opalescent, with craters	Ishimaru and Klos (1984)
MS	Colonies with red to orange color	Miller and Schroth (1972)

KB, King's B medium, SNA, sucrose nutritive agar; CCT, CCT medium; MS, Miller-Schroth medium.

2.1.2. API system

The strips of API 20E system were inoculated from a single colony suspension following the indications of the supplier. Each strip contained 20 standardized and miniaturized biochemical tests (*Figure 2.2-A*) which included the β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophane deaminase, indole production, acetoin production, gelatinase, glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, arabinose fermentation or oxidation. Results were read after 48 h of incubation at 25 ºC.

2.1.3. Biolog plates

Biolog GN plates (Biolog, Inc., Hayward, California, USA) identification system is based on 95 carbon sources utilization provided in a microtiter plate (*Figure 2.2-B*). Cultures were grown in BUG™ agar (*Annex 2*) for 24 h at 22 ºC. Bacterial suspensions in saline solution (0.85% NaCl) adjusted to 10^8 cuf·mL^{-1} were used. Biolog GN plates were then inoculated following the indications of the manufacturer. Plates were incubated at 25 ºC for 9 h before automatically reading at 405 nm of an optical density using a multiscan photometer operated with the ASCENT software (Ascent Research Edition Version 2.1, Labsystems, Helsinki, Finnland).

Figure 2.2. API 20E strip (**A**) and Biolog GN plate (**B**).

2.1.4. Statistical analysis

The results from the Biolog GN plates and from the API 20E system for each *E. amylovora* strain were subjected to cluster analysis. The similarity matrix between bacterial isolates was calculated using the Euclidean distance. Cluster analysis was performed with the Agglomerative Hierarchical grouping method through the cluster analysis procedure of S-PLUS (S-PLUS 6.0, Insightful Corporation, USA).

2.2. Serological identification by DASI-ELISA

The procedure previously described was used with 1 mL of pure culture suspension made on sterile distilled water (see *section 1.2*).

2.3. Molecular identification by PCR

Two types of PCR reactions were performed. A PCR using primers for the pEA29 plasmid (Bereswill *et al.,* 1992) and another for the *ams* gene (Bereswill *et al.,* 1995) as a confirmation test.

PCR directed to pEA29 plasmid (Bereswill *et al*., 1992) was performed as previously described in *Section 1.3*. The PCR directed to *ams* gene (Bereswill *et al*., 1995) was performed using another set of primers described in *Table 2.3*. PCR reaction was carried out in a volume of 50 µL, containing 5 µL of lysed cell suspension, 1 µL of dNTPs 10 mM (Amersham Pharmacia Biotech), 25 pmol of each primer, 1.5 µL Cl_2Mg 50 mM, 5 µL PCR buffer 10X, 35.1 µL of sterile demineralised water and 0.4 µL of Taq DNA polimerase 5 U·µL^{-1} (GIBCO). The PCR program consisted of an initial denaturation at 94 ºC for 5 min followed by 40 cycles of 94 ºC for 30 s, 52 ºC for 20 s, 72 ºC for 30 s, and a final elongation at 72 ºC for 10 min. Amplifications were performed with a GeneAMP® PCR system 9700 (PE Applied Biosystems).

Table 2.4. Size of the DNA amplified fragments obtained using different primers according to the annealing temperature

Target gene	**Primer**	**Sequence 5' → 3'**	**Temperature**[1] (ºC)	**Product**[2] (bp)	**Reference**
ams gene	AMSb1 A	GCTACCAGCAGGGTGAG	60	1600	Bereswill *et al.,* 1995
	AMSb1 B	TCATCACGATGGTGTAG			

[1]Annealing temperature corresponding to each pair of primers.
[2]Length of the DNA fragment amplified for each PCR.

The PCR products were separated by horizontal electrophoresis Mini-Sub® Cell GT (Bio-Rad, Barcelona, Spain) (*Figure 2.4-A*) on a 1.2% agarose gel in 1X TAE buffer (*Annex 1*) at 70 V for 45 min. The gel was stained with ethidium bromide for 30 min, and the PCR products were viewed with a UV transilluminator (*Figure 2.4-B*). *E. amylovora* strain PMV6076 was included as positive control while the negative control was realized with water. The colonies that generate an amplified product of the corresponding length were considered as strains of *E. amylovora*.

2.4. Pathogenicity test

Pathogenicity was tested using immature pear fruits obtained from a commercial orchard of cv. Passe Crassane. Fruits aged 6 weeks were collected in June and kept in the dark at 0 ºC to 4 ºC with moderate aeration and high relative humidity. Fruits were surface disinfected by immersion for 10 min in a solution of sodium hypochlorite (1%), washed two times by immersion in sterile distilled water, and let to remove excess water. Then fruits were wounded in the equatorial zone (four wounds per fruit) with a nail tip to an approximate depth of 5 mm and approximate wide of 3 mm. Fruits were placed on polystyrene tray packs which were placed in boxes.

Strains were cultured overnight at 25 ºC in Luria Bertani (LB) agar plates (*Annex 2*). Colonies were scraped from the agar surface and suspended in sterile distilled water. The cell concentration was adjusted turbidimetrically to 10^7 cfu·mL^{-1}. Immature pear fruits were inoculated by placing 10 µL of a 10^7 cfu·mL^{-1} pathogen suspension in each wound. The boxes were hermetically closed with plastic bags to maintain high humidity conditions and biosafety. Inoculations were performed in a class II biological safety cabinet (Nuarie class II UN-426-400E, USA). Finally, fruits were maintained at 21 ºC in an expression chamber. The incidence of infections was assessed after 8 days of pathogen inoculation. Wounds were considered infected when drops of bacterial exudates or necrosis were detected in and around them (*Figure 2.3*).

Figure 2.3. Symptoms observed in immature pear fruits after infection by *E. amylovora*. Oozes (**A**), necrosis (**B**) or necrosis with oozes (**C**).

2.5. Genetic diversity by RFLP fingerprinting

The genetic diversity of *E. amylovora* strains was studied by RFLP analysis of the *ams* gene and Macrorestriction Fragment Length Polymorphism analysis (MRFLP) resolved by PFGE.

2.5.1. Bacterial strains

A selection of 12 strains of *E. amylovora* (EAZ4, EPS101, CFBP1430, IVIA1614.2, NCPPB595, NCPPB3159, OMP-BO1185, PMV6076, UPN500, UPN546, UPN611 and USV1000) was studied, based on origin (host plant, location, year of isolation) (*Table 2.2*).

2.5.2. RFLP fingerprinting of *ams* gene

Strains were grown on LB agar at 23 ºC for 24 h. Then, the DNA was extracted using a commercial extraction kit (Wizard® Genomic DNA Purification Kit. Promega, Madison, USA). PCR amplification of *ams* gene (Bereswill *et al.,* 1995) was performed using the primer set of AMSb1A and AMSb1B as described previously (*section 2.3*). Ten microliters of the PCR products were separated by horizontal electrophoresis on a 1.5% agarose gel in 1X TAE buffer (*Annex 1*) at 70 V for 45 min. The gel was stained with ethidium bromide for 30 min, and the PCR products were visualized with a UV transilluminator. Cleaned PCR products were obtained after electrophoresis and for this reason it was not necessary to purify the expected band. Ten microliters of the PCR products were digested with 0.8 µL of the restriction

enzymes *Mlu* I, *Rsa* I and *Msp* I (Roche, Barcelona, Spain) at 10 $U \cdot \mu L^{-1}$ in a total volume of 15 µL that contains 2.7 µL of sterile mili-Q water and 1.5 µL of the corresponding restriction buffer. The digestions were performed at 25 ºC overnight. Separation of digested products in 2.5% agarose gels was performed as described above. The gel was digitalized using a digital camera (DC120 Zoom Digital Camera, Eastman Kodak Company, NY, USA) and the patterns were analyzed by 1D Image Analysis System 120 software (Eastman Kodak Company, NY, USA).

2.5.3. PFGE analysis of genomic DNA

MRFLP analysis of genomic DNA based on the protocol described for *P. fluorescens* (Badosa, 2001) and the protocol described for *E. amylovora* (Zhang and Geider, 1997) was used to characterize the genetic diversity of *E. amylovora* strains. Bacterial cultures were grown on LB broth at 25 ºC until an optical density of 0.6 to 1.0 at 620 nm. Then, 5 mL aliquots of broth cultures were centrifuged, and the bacterial cells were washed three times with PET IV buffer (*Annex 1*). The cleaned bacterial pellet was suspended in 2 mL of TE buffer (*Annex 1*) and 2 mL of 2% ultrapure agarose (InCert® Agarose, RMC® Bioproducts, Maine, USA) and pipetted into a plastic mould. Each plug has a volume of 100 – 110 µL. After 15 min at 4 ºC the hardened agarose plugs were immersed in digestion buffer (*Annex 1*) for 48 h at 56 ºC and then soaked three times in TE buffer for 30 min. A quarter of plug (approximately 33 µL) was digested overnight at 37 ºC in a total volume of 200 µL, containing 143.5 µL of sterile milli-Q water, 20 µL of restriction buffer H, 1 µL of BSA, and 2.5 µL of *Xba* I enzyme (10 $U \cdot \mu L^{-1}$).

Strains of *E. amylovora* were differentiated by PFGE after digestion of genomic DNA with rare cutting restriction enzymes. After digestion, the resulting DNA fragments were resolved by PFGE analysis with Contour-clamped Homogeneous Electric Field (CHEF) system (Gene Navigator SQ, Pharmacia Biotech, Spain) in a Hepes buffer (*Annex 1*) for 22 h at 14 ºC with linear ramping at 200 V and 120 mA in a 1.2% agarose gel (Sea-Kem®, RMC® Bioproducts, Maine, USA). The linear ramping time was 5 to 25 s. The electrophoresis gel was stained with ethidium bromide for 30 min and visualized with a UV transilluminator. Image was captured by digital camera and analysed with 1D Image Analysis System 120 software.

The RFLPs obtained with *Xba* I digested DNAs were used to group strains from different geographic regions and host plant. According to Zhang and Geider (1997) and Zhang *et al*. (1998), four patterns for *Xba* I digest have been described in relation to the absence, shift or lack of one of the bands: *Pt1, Pt2, Pt3* and *Pt4* (*Figure 2.4*). The first defined pattern was the *Pt1*, and was assigned to most strains from central Europe. This pattern was characterized by 16 bands. Pattern *Pt2* was observed for the Mediterranean strains but was also found for some American strains and differ from *Pt1* in a band that shift from 220 to 210 Kb. Pattern *Pt3* was defined for French and Belgium strains and is characterized for be devoid of the 130 Kb band. Finally, pattern *Pt4* was designed for strains from Spain but can also be observed in some strains from England and West of France. This pattern is quite similar to *Pt1* but there is a slightly smaller distance between the two bands of 190 and 220 Kb (Jock *et al.*, 2002).

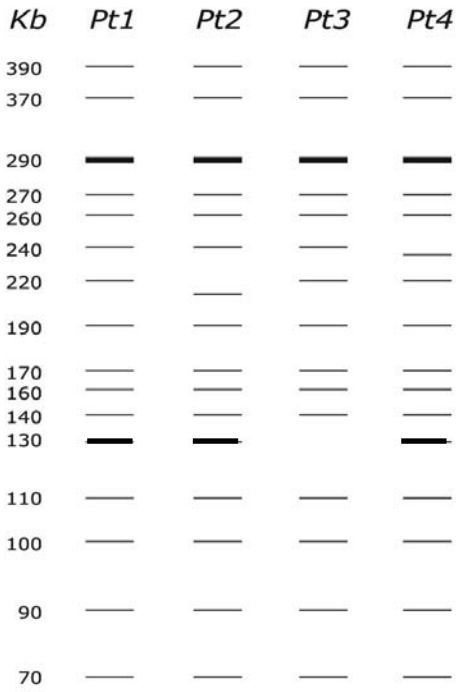

Figure 2.4. Scheme of PFGE patterns of *E. amylovora* obtained from *Xba* I digestion of genomic DNA as described by Zhang *et al.,* 1998.

3. Virulence of strains

Virulence of *E. amylovora* strains was studied on the basis of disease progression curves and dose-response experiments in inoculated immature pear fruits. The virulence experiments were performed following the same procedure described to perform the pathogenicity test (see *section 1.2*).

3.1. Effect of the *E. amylovora* concentration on infection incidence. Dose-response relationships

Preliminary assays were performed in immature pear fruits with a reduced group of strains to establish the optimal pathogen concentrations for virulence determination. The first assay was performed with 18 strains of *E. amylovora* (UPN529, UPN597, UPN536, UPN588, UPN549, UPN601, UPN562, UPN504, UPN546, UPN605, UPN599, UPN609, UPN513, UPN575, UPN611, EPS101, CUCM273 and PMV6076) (*Table 2.2*). Inoculations were performed with 10 µL of bacterial suspensions at 10^6 or 10^7 cfu·mL^{-1}. The methodology was the same described in *Section 2.4*. The experimental design consisted of three repetitions of 6 fruits per repetition (four wounds per fruit) for each strain and concentration. The incidence of infections was calculated as infected wounds per total wounds inoculated for each repetition, and assessed after 9 days of incubation. ANOVA was performed to test the effect of strain and dose in the incidence of infections. Means were separated using the Tukey's test with a significance of 0.05.

In a second experiment the effect of pathogen concentration on the incidence of infections in immature pear fruits was determined with a dose-response assay. Six strains of *E. amylovora* (IVIA1614-2, NCPPB595, UPN529, UPN611, EPS101 and NCPPB311) (*Table 2.2*), were studied at several concentrations (6, 60, $6 \cdot 10^2$, $6 \cdot 10^3$, $6 \cdot 10^4$, $6 \cdot 10^5$, $6 \cdot 10^6$ and $6 \cdot 10^7$ cfu·mL^{-1}). The experimental design consisted of three repetitions of 6 fruits per repetition (four wounds per fruit) for each strain and concentration. The incidence of infections was assessed at 0, 2, 3, 4, 5, 6, 7 and 10 days after inoculation.

Infection incidence data at each pathogen concentration were used to estimate virulence parameters with a non-linear regression procedure using the probit and hyperbolic saturation models (Montesinos and Bonaterra, 1996). The probit model provides parameters such as the median effective dose (ED_{50}), and the pathogen efficiency (τ). The hyperbolic saturation model provides the maximum incidence of infections (Y_{max}) and the ED_{50}. Equations are as follow:

Probit model: $$y = \phi\left[\frac{(\log 10(x) - \lambda)}{\tau}\right]$$

where ϕ denotes the cumulative distribution function for the standard normal. The parameter λ is equivalent to ED_{50} and τ is the efficiency of the pathogen.

Hyperbolic saturation model: $$y = y_{\max}\frac{x}{(x + K_x)}$$

where the parameter Y_{max} is the maximum disease incidence proportion while the K_x is a half saturation constant corresponding to ED_{50}.

Moreover, we estimated the minimum infective dose of pathogen (*MID*). The *MID* was calculated considering the minimum incidence detectable according to the experimental design used which corresponded to a single wound infected out of 24 inoculated wounds (P=0.0416).

Estimation of parameters (ED_{50} and *MID*) was performed by non-linear and by linear regression methods. Parameter estimation of the probit and the hyperbolic saturation models by non-linear regression was performed with the Gauss-Newton method that uses the first derivative of the independent variable for each parameter (Draper and Smith 1982; SAS Institute, 1989). Previously to the iteration process, it was necessary to start with candidate initial values for model parameters to a logical value. The goodness of fit of the observed values to the predicted values (coefficient of determination R^2) and the significance (P>F) was determined for each pathogen strain and concentration. The statistical analysis was performed using the JMP for Machintosh (JMP 2.0; SAS Institute Inc., Cary NY, USA).

Parameters estimation by linear regression was performed using infection incidence on pathogen concentrations. Infection incidence was transformed to probit for the probit model. Regression lines obtained were evaluated by the coefficient of determination (R^2) and the

significance ((P>F) < 0.05). The regressions and the significance calculation were performed using the JMP for Macintosh.

3.2. Disease progression curves

The virulence of the 53 strains of *E. amylovora* described in the *Table 2.2* was evaluated in immature pear fruits using disease progression curves. The strains were inoculated in immature pear fruits at 10^3 or 10^5 cfu·mL^{-1}. The experimental design consisted of three repetitions of 6 fruits per repetition for each strain and concentration. Virulence was determined on the basis of the incidence of infections. Incidence was assessed at 0, 3, 4, 5, 6, 7, 8, 9 and 10 days after the pathogen inoculation.

The following kinetic parameters calculated from disease progression curves were used as a measure of the virulence: maximum disease proportion (k), rate of disease progression (r_g), time of start of infection (t_0), and area under the disease progression curve (*AUDPC*). These parameters were grouped in two groups: (1) indicating infectivity potential (t_0 and k), and (2) related to aggressiveness (r_g). Infectivity was considered as the ability of the strains to initiate an infection and to produce infections, and aggressiveness as the rapidity in developing symptoms and disease progression.

The k and *AUDPC* were determined directly from disease progression curves. K was determined as the incidence at the end of the incubation period. *AUDPC* was calculated using the following formula:

$$AUDPC = \sum_{i}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) \cdot (t_{i+1} - t_i)$$

where y is the incidence of infections for the i observation at time t.

Several models were tested to fit data of disease progression curves, like the exponential, monomolecular, logistic, Gompertz and modified Gompertz (*Table 2.5*). The modified Gompertz model was selected to account for the typical delay observed in the start of infections depending on the strain.

Parameter estimation using the modified Gompertz model was performed by a non-linear regression procedure of disease incidence on time by means of Gauss-Newton method that uses for convergence the first derivative of the dependent variable for each parameter (Draper and Smith, 1982; SAS Institute, 1989). Previously to start iterations, it was necessary to fix candidate values for the K for each experiment, as well as initial values for iteration model parameters (B_g, r_g and t_0). Initiate values were for B_g = 10, r_g = 1 and t_0 to the approximated observed value. For each curve, the goodness of fit of the observed values to the predicted values (coefficient of determination R^2) and the significance were determined (P>F). The statistical analysis was performed using the JMP for Macintosh.

Table 2.5. Models used for the analysis of disease progression curves (modified from Madden and Campbell, 1990)

Model	Function	Lineal form	Integration constant (B)
Exponential	$y=y_0 \cdot \exp(r_e \cdot t)$	$\ln(y)=\ln(y_0)+r_e \cdot t$	------
Monomolecular	$y=k \cdot [1-B_m \exp(-r_m \cdot t)$	$\ln(k/k-y)=-\ln(B_m)+r_m \cdot t$	$B_m=-\ln[(k-y_0)/k]$
Logistic	$y=k/[1+\exp(-(B_l+r_l \cdot t))]$	$\ln[y/(k-y)]=\ln(y_0/(k-y_0))+r_l \cdot t$	$B_l=-\ln[y_0/(k-y_0)]$
Gompertz	$y=k \cdot \exp[-B_g \cdot \exp(-rg \cdot t)]$	$-\ln[-\ln(y/k)]=-\ln(B_g)+r_g \cdot t$	$B_g=-\ln(y_0/k)$
Modified Gompertz	$y=k \cdot \exp[-B_g \cdot \exp(-rg(t-t_0)]$	$-\ln[-\ln(y/k)]=-\ln(B_g)+r_g \cdot t-r_g \cdot t_0$	$B_g=-\ln(y_0/k)$

K: maximum disease level or curve asymptote
r: rate of disease progression
t_0: time of start of infections
y: disease level (from 0 to 1)
y_0: initial disease level

ANOVA was performed with all the estimated parameters, to test for the effect of strain and dose. Means were separated by the Ryan-Einot-Gabriel-Welsh Multiple Range test at $P \leq 0.05$ and results used to group strains according to their virulence. The analysis was performed with the GLM procedure of the PC-Statistical Analysis System version 8.2 (SAS Institute Inc., Cary, NY, USA).

Strains were classified on the basis of disease progression parameters at 10^5 cfu·mL^{-1} by means of grouping according to the arrangement obtained by the means separation test. Strains were clustered arbitrarily in three groups: high, moderate and low virulence.

However, in order to have a virulence classification according to all the parameters jointly, strains were classified by means of a composite virulence index (*CVI*) calculated using the following equation:

$$CVI = \frac{(\tau_g + t_0 + k + AUDPC)}{12}$$

where each parameter is considered as 3 when strains presents high, 2 for moderate, and 1 for low values. Thus, the maximum attainable virulence index is 1 and the minimum 0.

4. Optimization of model pathosystems for interaction studies

Two model systems were optimized during this study, the pear blossom assay and the young shoot assay. These systems were optimized to perform studies of interaction between the host, the pathogen and the potential biocontrol agents. The more important aspect to take into account was to determine the pathogen concentration to get sufficiently high levels of disease with low variability, but suitable to get good levels of biological control.

4.1. Pear blossom model

The pear blossom model was based on the crab apple blossom assay described by Pusey (1997). Two methods of inoculation and two pathogen concentrations were assessed in order to select the suitable methodology.

Individual pear flowers were obtained from detached pear branches and forced to bloom in an environmental chamber (Montesinos and Vilardell, 1991). Pear branches with flower buds of Doyenne de Comice cultivar were taken from a commercial orchard in Girona. Branches aged 2 years, of 40 to 60 cm length, each containing 7-15 flower buds, were cut in February 2001 at the dormant bud development stage. Branches were covered with plastic bags and the cut ends were immersed immediately in water, transported to the laboratory, and stored at 6 ºC in the dark for about 2 months. Just before force blooming, branches were surface disinfected by washing with a sodium hypochlorite (1%) solution, their basal ends pruned diagonally while submerged in water to avoid occlusion of vascular system by air, and placed in small containers with 200 ml of a sterilized 10% sucrose solution. The method used to force blooming was a modification of a method already described (Szkolnik and Hickey, 1986). Branches were placed in an environmental chamber at 22-25 ºC, with 16 h of fluorescent light and 70 to 80% relative humidity (*Figure 2.5*).

Periodically, the base end was pruned and the sucrose solution was replaced to avoid yeast growth and occlusion of the vascular system. Under these conditions, the bloom occurred in about 2-3 weeks depending on the previously accumulated chilling hours. The open blossoms were detached from branches and the individual flowers were maintained with the cut peduncle submerged in 1 mL of 10% sucrose solution in a single plastic Eppendorf vial of 1.5 mL. The sucrose concentration used permitted flowers enclosed in plastic containers to maintain a healthy appearing state with stigmas still green and petals retained for 5 to 6 days. The vials were placed in tube racks until use.

Figure 2.5. Pear branches with dormant flower buds forced to bloom by immersion on a sterilized 10 % sucrose solution at 21 ºC, 70-80 % relative humidity and 16 h of light. **A**: early stage; **B**: advanced stage.

Two inoculation methods were assessed in detached flowers. The first method was based on rubbing the pistil with a socked paintbrush in a pathogen suspension. The second was based on depositing a 10 μL drop of the pathogen suspension on the hypanthium. Besides, both inoculation methods were tested at two pathogen concentration, 10^7 cfu·mL^{-1} and 10^8 cfu·mL^{-1}. Inoculation was done in a class II biological safety cabinet. Flowers were covered with plastic bags and were incubated in an expression chamber at high relative humidity with 16 h of fluorescent light at 21 ºC. Each treatment consisted of three repetitions with 8 flowers per repetition. Methods were compared according to the disease incidence (*I*) and severity (*S*) after 8 days of incubation.

Disease incidence was assessed by means of the following equation:

$$I = \left(\frac{F_i}{F_t} \right) \cdot 100$$

where *I* is the incidence, F_i the number of infected flowers, and F_t is the number of total flowers inoculated.

Disease severity was calculated using the following equation:

$$S = \sum_{i \to 1}^{i} \left(\frac{SI_i}{n \cdot 3} \right) \cdot 100$$

where *S* is the disease severity, *SI* the severity index, *i* is the flower number, *n* the total flowers and *3* the maxim severity index.

Severity indexes (*SI*) were determined for each flower according to a scale from 0 to 3. Flowers were considered infected when necrosis was detected in the pistil. Severity indexes were described in function of symptoms observed: 0, no symptoms; 1, partial pistil necrosis; 2, total pistil necrosis, and 3, necrosis progression through peduncle (*Figure 2.6*).

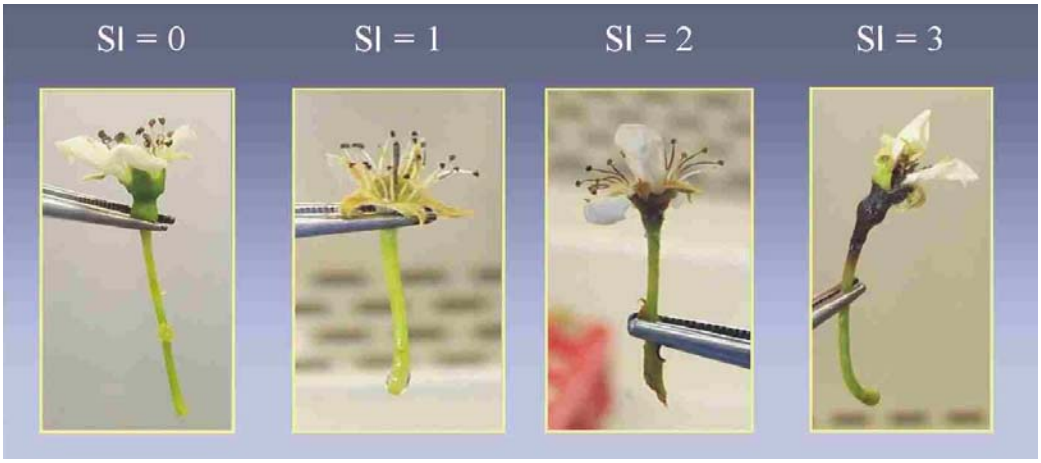

Figure 2.6. Severity Index (*SI*) of fire blight in detached flowers. Levels: 0, no symptoms observed; 1, partial pistil necrosis; 2, total pistil necrosis; 3, necrosis progression through peduncle.

ANOVA was performed to test the effect of inoculation method and the pathogen application dose on the incidence and severity of blossom blight. The means were separated according to the Tukey's method ($P \leq 0.05$).

4.2. Young pear shoot model

The young pear shoots model was based on the methodology described by Tharaud *et al*. (1997), but instead of trees in pots, detached pear branches containing vegetative buds were used. In the present work two pathogen concentrations were assessed.

Young shoots with vegetative buds of the cultivar Doyenne du Comice obtained from detached branches from a commercial orchard of Girona were used. Branches aged 2 years, 40 to 60 cm length and each one containing from 7 to 15 buds, were cut in February 2001 at the development stage of dormant buds. The same procedure used to force blooming was used (*section 2.2.4*). The branches were cut in pieces around 15 cm long with three shoots each one. Then, cut branches were submerged in a sterilized 10% sucrose solution (*Figure 2.7-A*). The three youngest expanded leaves of each shoot were wounded by a double incision (~1mm) perpendicular to the midrib, approximately in the middle of the leaf. One drop (10 μL) of pathogen suspension was immediately placed on the wound.

The pathogen concentrations assessed were 10^6 or 10^7 cfu·mL^{-1}. Sterile water was used as a non-treated control. Inoculations were done under biological safety conditions (*Figure 2.7-B*). Branches were hermetically closed in plastic bags and incubated in an expression chamber at 21 ºC, high relative humidity and 16 h of fluorescent light (*Figure 2.7-C*).

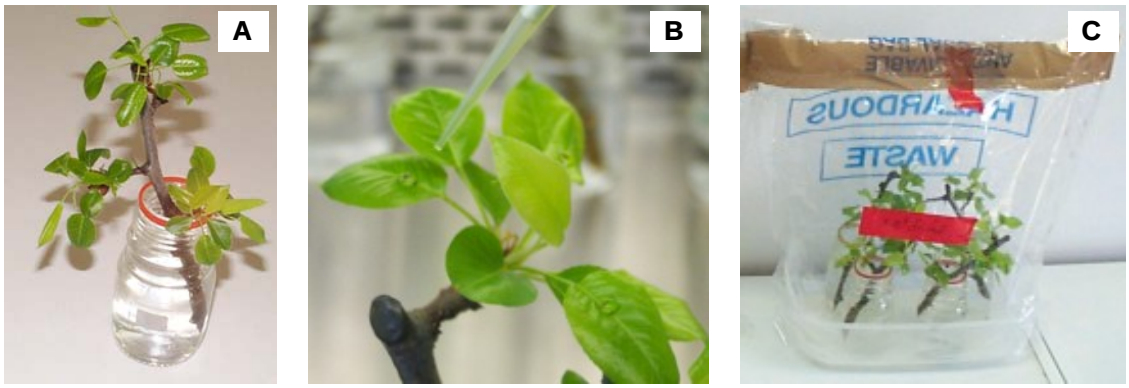

Figure 2.7. Detached shoot assay procedure. Cut branch with young shoots submerged in sucrose solution (**A**). Inoculation of pathogen by the deposition of *E. amylovora* suspension at 10^7 cfu·mL^{-1} on the leaf wounds (**B**). Branches with young shoots covered with plastic bags during pathogen incubation (**C**).

The experimental design consisted of three repetitions with 3 branches per repetition. Each branch contained two or three shoots, and three leaves per shoot were inoculated. Disease incidence and severity was evaluated for each branch. Evaluation was performed after 10 days of incubation by means of the equations previously described (*section 4.1*). Severity was determined for each leaf according to a scale from 0 to 3. Leaves were considered

infected when necrosis was detected around the wound. Severity indexes were described in function of symptoms observed: 0, no symptoms; 1, necrosis around midrib; 2, total midrib necrosis, and 3, necrosis progression through petiole (*Figure 2.8*). The effectiveness of the treatments was determined in relation to the maximum attainable incidence and severity.

Figure 2.8. Severity Index (*SI*) used in the shoot blight assay. Severity Index levels: 0, without symptoms; 1, necrosis around midrib; 2, total midrib necrosis; 3, necrosis progression through petiole.

ANOVA was performed to test the affect of pathogen concentration on incidence and severity of shoot blight. The means were separated according to the Tukey's test ($P \leq 0.05$).

RESULTS

1. Build-up of a collection of *E. amylovora* strains

Only one *E. amylovora* isolate was detected and isolated from the symptomatic samples processed from different commercial orchards of pear and apple trees, and from different ornamental plant nurseries of Catalunya. Suspect isolate was first detected in enriched plant macerate by DASI-ELISA from a symptomatic pear shoot taken from a commercial orchard (cv. Conference) of Lleida. The strain also presented a typical dome shaped colony morphology growth on SNA and white colony on KB, and was identified as *E. amylovora* by PCR detection using pEA29 primers and its pathogenicity in immature pear fruits producing the typical *E. amylovora* infection with necrosis and exudates production. This strain was coded as EPS101.

2. Characterization of a diverse collection of *E. amylovora* strains

2.1. Identification and pathogenicity test

The results of characterization by growth on different media, PCR using different primers (*Figure 2.9*), enrichment DASI-ELISA, and level of pathogenicity in immature pear fruits of the 53 *E. amylovora* strains from the collection are shown in *Table 2.5*. Great homogeneity

among *E. amylovora* strains was observed for cultural characteristics and PCR and ELISA methods. Only 5 out of 53 strains showed differential serological response (negative for ELISA) and PCR amplification using pEA29 primers. Greater variability was observed in immature pear fruit test, and strains could be classified according to their degree of virulence in high, moderate and low virulent.

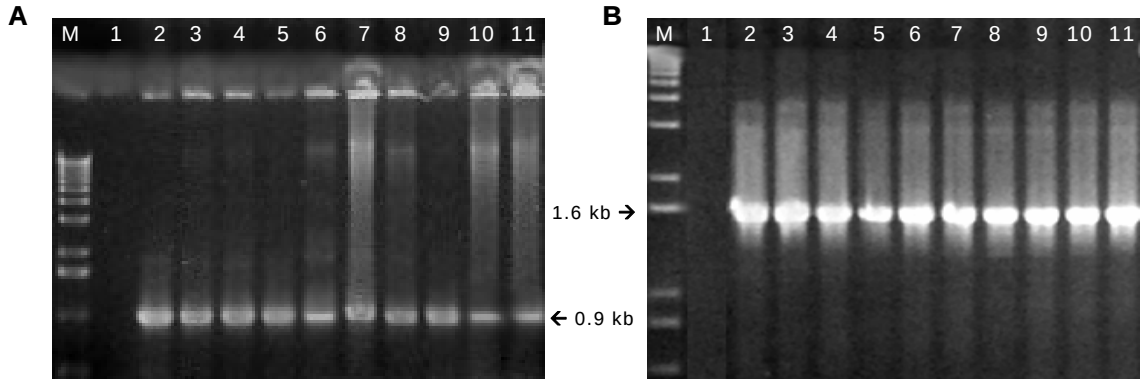

Figure 2.9. Agarose gel electrophoresis corresponding to pEA29-PCR (Bereswill *et al*., 1992) (~900 bp) (**A**) and *ams* gene-PCR (Bereswill *et al*., 1995) amplification products (~1600 bp) (**B**). Lanes 1 to 12, negative control, strain EPS101, USV1000, NCPPB595, CFBP1430, PMV6076, Ea115.22, UPN500, IVIA1614.2, UPN611 and EAZ4; M, 1 Kb plus DNA ladder.

Table 2.6. Growth on different media[x] (KB, SNA, MS and CCT), PCR[y] using pEA29 plasmid and *ams* gene primers, DASI-ELISA, and intensity of infections in immature pear fruits (IPF) of 53 *E. amylovora* strains

Strain	Culture media				PCR		ELISA	IPF test[z]
	KB	SNA	MS	CCT	pEA29	*ams* gene		
EPS100	+	+	+	+	+	nd	+	h
EPS101	+	+	+	+	+	+	+	h
USV1000	+	+	+	+	+	+	+	h
USV1043	+	+	+	+	+	nd	+	h
USV2194	+	+	+	+	+	nd	+	m
USV2773	+	+	+	+	+	nd	+	h
USV4300	+	+	+	+	+	nd	+	h
USV4320	+	+	+	+	+	nd	+	h
USV4408	+	+	+	+	-	nd	+	h
USV4496	+	+	+	+	+	nd	+	h
USV4499	+	+	+	+	+	nd	+	h
USV4500	+	+	+	+	+	nd	+	h
USV4501	+	+	+	+	+	nd	+	h
USV4512	+	+	+	+	+	nd	+	h
USV4576	+	+	+	+	+	nd	+	h
CUCM273	+	-	+	+	+	nd	-	h
NCPPB311	+	+	+	+	+	nd	+	l
NCPPB595	+	+	+	+	+	+	+	m
NCPPB683	+	+	+	+	+	nd	+	h
NCPPB1734	+	+	+	+	-	nd	-	m
NCPPB1819	+	+	+	+	+	nd	+	h
NCPPB2080	+	+	+	+	+	nd	+	h

(Continue)

Strain	Culture media				PCR		ELISA	IPF test[z]
	KB	SNA	MS	CCT	pEA2	*ams* gene		
NCPPB2791	+	+	+	+	+	nd	+	h
NCPPB3159	+	+	+	+	+	nd	+	h
NCPPB3548	+	+	+	+	+	nd	+	m
CFBP1430	+	+	+	+	+	+	+	h
PMV6076	+	+	+	+	+	+	+	l
OMP-BO1185	+	+	+	+	+	+	+	h
IVIA 1614.2	+	+	+	+	-	+	+	h
Ea115.22	+	+	+	+	+	+	+	l
UPN500	+	+	+	+	+	+	+	h
UPN504	+	+	+	+	+	nd	-	h
UPN506	+	+	+	+	+	nd	+	h
UPN513	+	+	+	+	+	nd	+	h
UPN514	+	+	+	+	u	nd	-	h
UPN524	+	+	+	+	+	nd	+	l
UPN529	+	+	+	+	+	nd	+	h
UPN530	+	+	+	+	-	nd	-	h
UPN536	+	+	+	+	+	nd	+	h
UPN544	+	+	+	+	+	nd	+	h
UPN546	+	+	+	+	+	nd	+	h
UPN562	+	+	+	+	+	nd	+	h
UPN575	+	+	+	+	+	nd	+	h
UPN576	+	+	+	+	+	nd	+	h
UPN596	+	+	+	+	+	nd	+	h
UPN588	+	+	+	+	+	nd	+	h
UPN597	+	+	+	+	+	nd	+	h
UPN610	+	-	+	+	e	nd	+	l
UPN611	+	+	+	+	+	+	+	h
EAZ1	+	+	+	+	+	nd	+	h
EAZ4	+	+	+	+	+	+	+	m
EAZ7	+	+	+	+	+	nd	+	h
EAZ9	+	+	+	+	+	nd	+	m
EAZ13	+	+	+	+	+	nd	+	m

[x]KB, King's B medium; SNA, sucrose nutritive agar; MS, Miller-Schroth medium; CCT, CCT medium; +, typical growth; -, strain does not growth or not growth as a typical *E. amylovora*.
[y]u, unspecific bands; e, amplification products larger than the expected; nd, not done.
[z]Immature pear fruit test according to the maximum incidence of infections observed after 5 days of inoculation with 10 µL of an *E. amylovora* suspension at 10^5 cfu·mL^{-1}; h, high; m, moderate; l, low virulence.

Strains were also characterized with the API 20E and Biolog GN systems. In both cases few differences were also observed. For API 20E system, only 8 tests out of 20 corresponding to arginine dihydrolase, citrate, mannitol, sorbitol, rhamnose, sucrose, melibiose, and amygdalin use gave different response (*Table 2.7*). The remaining tests were either positive or negative for all strains. Aggregation analysis using API system resulted in dendrogram (*Figure 2.10*), Seven groups were arbitrarily defined in the aggregation analysis at an Euclidean distance of 1.0 (*Table 2.8*). Forty three out of fifty three strains (81%) were included in only two groups while the rest of the groups included a small percentage of strains. The first group included fourteen strains (26%) and the second group included twenty nine strains (55%). Apparent relations according to the grouping were not observed.

Table 2.7. Tests of API 20E system[x] grouping strains of *E. amylovora* at an Euclidean distance of 1.0

Group	Number of strains	ADH	CIT	MAN	SOR	RHA	SAC	MEL	AMY
1	14	-	-	+	+	-	+	-	-
2	29	-	-	+	+	-	+	+	-
3	3	-	-	-	+	-	+	+	-
4	3	-	+	+	+	-	+	+	-
5	1	+	-	+	+	+	+	-	+
6	1	-	-	-	-	-	-	+	-
7	2	+	+	+	+	+	+	+	+

ADH, arginine dihydrolase; CIT, citrate use; MAN, SOR, RHA, SAC, MEL and AMY, use of mannitol, sorbitol, rhamnose, sucrose, melibiose, and amygdalin, respectively.

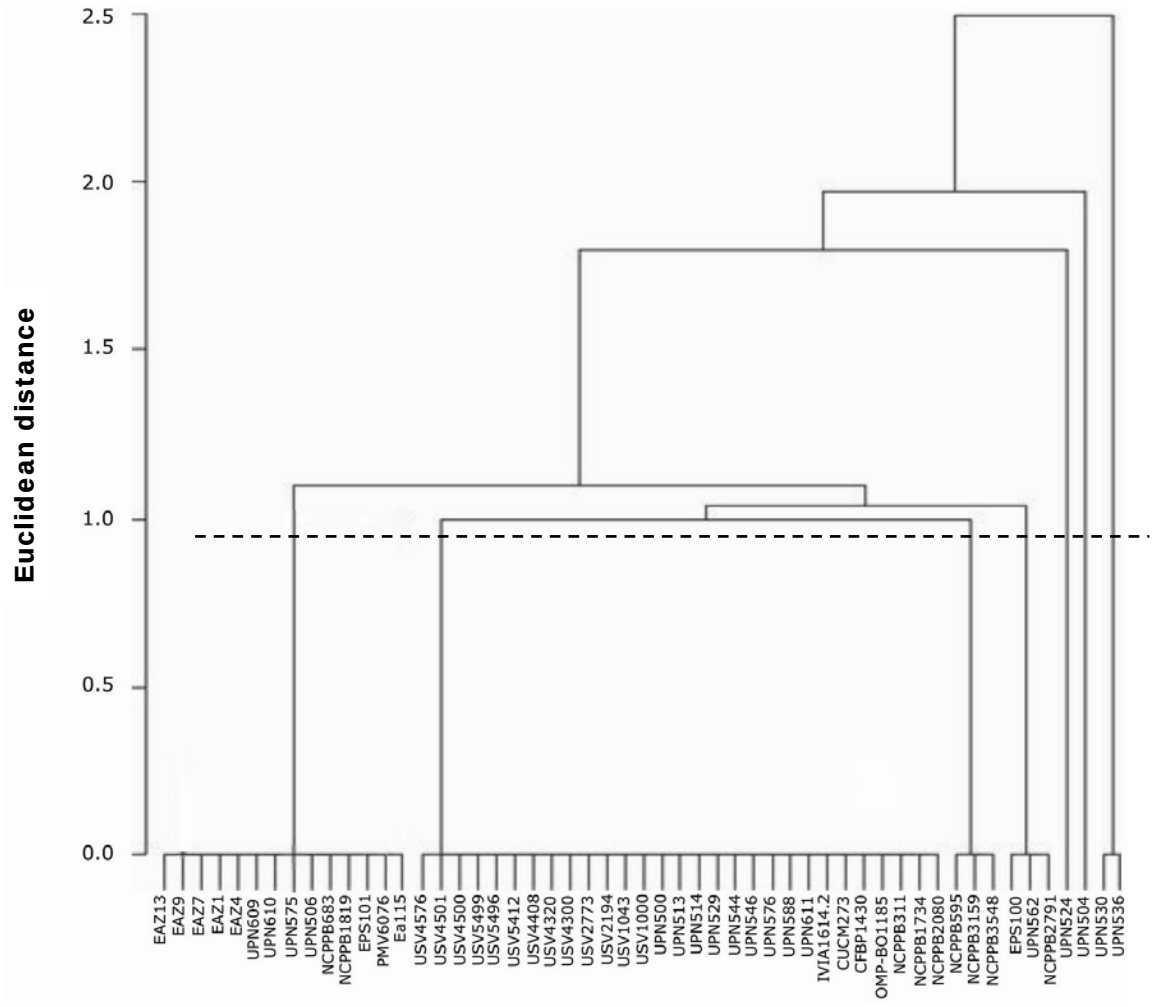

Figure 2.10. Dendrogram indicating the relatedness between the strains listed in *Table 2.2* as determined using API system data. Euclidean distances were calculated on the basis of the differences between profiles. These data were then clustered using the agglomerative hierarchical method. Dotted line indicates the Euclidean distance to obtain the groups described in *Table 2.6*.

Table 2.8. Grouping of *E. amylovora* strains situated at an Euclidean distance of 1.0 according to API 20E system

Group	Strains
1	EAZ13, EAZ4, EAZ7, EAZ4, UPN609, UPN610, UPN575, UPN506, NCPPB683, NCPPB1819, EPS100, PMV6076, Ea115.22
2	USV1000, USV1043, USV2194, USV2773, USV4300, USV4320, USV4408, USV4496, USV4499, USV4500, USV4501, USV4512, USV4576, UPN500, UPN513, UPN514, UPN529, UPN544, UPN546, UPN576, UPN588, UPN611, CUCM273, CFBP1430, OMP-BO1185, NCPPB311, NCPPB1734, NCPPB2080
3	NCPPB595, NCPPB3159, NCPPB3548
4	EPS100, UPN562, NCPPB2791
5	UPN524
6	UPN504
7	UPN530, UPN536

For Biolog GN system, only 25 tests out of 94 carbon sources corresponding to L-Serine, D-Galactose Cel·lobiose, Psicose, Timidine, D-Raphinose, β-Methyl-D-Glucoside, L-Arabinose, m-Inositol, Gentiobiose, D,L-Carnitine, β-cetoglutaric acid, Uridine, L-alanil-Glycine, L-Aspartic acid, glycil-L-Aspartic acid, L-Alanine, D,L-α-Glicerol-phosphate, L-Aspargine, D-Gluconic acid, methyl-Piruvate, Alaninamide, L-Proline, D,L-Lactic acid, D-Manose gave differential response (*Table 2.9*). The remaining tests were either positive or negative for all strains. Aggregation analysis using Biolog GN system (*Figure 2.11*) defined 11 groups at Euclidean distance of 1.75 (*Table 2.10*), but 38 out of 53 strains (72%) were included in the first group.

Table 2.10. Grouping of *E. amylovora* strains situated at an average distance of 1.75 according to Biolog GN system

Group	Strains
1	EPS100, EPS101, USV1000, USV1043, USV2194, USV2773, USV4300, USV4320, USV4408, USV4496, USV4499, USV4500, USV4501, USV4512, USV4576, NCPPB595, NCPPB683, NCPPB1734, NCPPB3548, PMV6076, OMPBO1185, Ea115.22, UPN500, UPN504, UPN506, UPN513, UPN514, UPN524, UPN529, UPN530, UPN536, UPN544, UPN575, UPN576, UPN609, UPN610, EAZ1, EAZ4, EAZ9,
2	CUCM273, CFB1430, UPN546, IVIA1614.2
3	NCPPB311
4	UPN588, UPN611
5	EAZ7
6	NCPPB2791
7	NCPPB1819
8	EAZ13
9	NCPPB2080, NCPPB3159
10	UPN562
11	UPN513

Table 2.9. Biolog GN tests associated to *E. amylovora* groups defined by the Euclidean distance of 1.7

Group	Number of strains	L-Serine	D-Galactose	Cel.lobiose	Psicose	Timidine	D-Raphinose	β-Methyl-D-Glucoside	L-Arabinose	m-Inositol	Gentiobiose	D,L-Carnitine	α-cetoglutaric acid	Uridine	L-alanil-Glycine	L-Aspartic acid	glycil-L-Aspartic acid	L-Alanine	D,Lα-Glicerolphosphate	L-Aspargine	D-Gluconic acid	methyl-Piruvate	Alaninamide	L-Proline	D,L-Lactic acid	D-Manose
1	38	73[y]	86	11	8	8	3	97	3	-	+	+	8	84	92	97	86	95	-	-	+	89	11	97	3	3
2	4	+	75	25	-	25	-	+	25	50	+	+	-	75	+	+	75	+	-	-	+	+	-	+	-	-
3	1	-	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	-	-	+	+	-	+	-	-
4	2	+	50	-	-	-	-	+	-	-	+	+	-	50	+	+	+	+	-	-	+	+	-	+	-	-
5	1	+	+	-	-	-	-	+	-	-	+	+	-	+	+	+	+	+	-	-	+	+	+	+	-	-
6	1	+	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-
7	1	+	+	-	-	-	-	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	-	+	-	-
8	1	-	+	-	-	-	-	+	-	-	+	+	-	+	+	+	+	+	-	-	+	+	+	+	-	-
9	2	50	+	-	-	-	50	+	-	-	+	+	-	-	+	50	-	50	-	-	50	+	-	+	-	-
10	1	+	-	-	-	+	-	+	-	-	+	+	+	+	+	+	+	+	-	-	+	+	-	+	-	-
11	1	+	+	-	-	-	-	+	-	-	+	+	-	+	+	+	+	+	-	-	+	+	-	+	-	-

[x]Biolog GN plates are composed of 95 carbon sources. In the table there are the carbon sources that were used differentially by *E. amylovora* strains.
[y]Test response; +, all the strains were positive; -, all the strains were negative; number, percentage of strains that were positive.

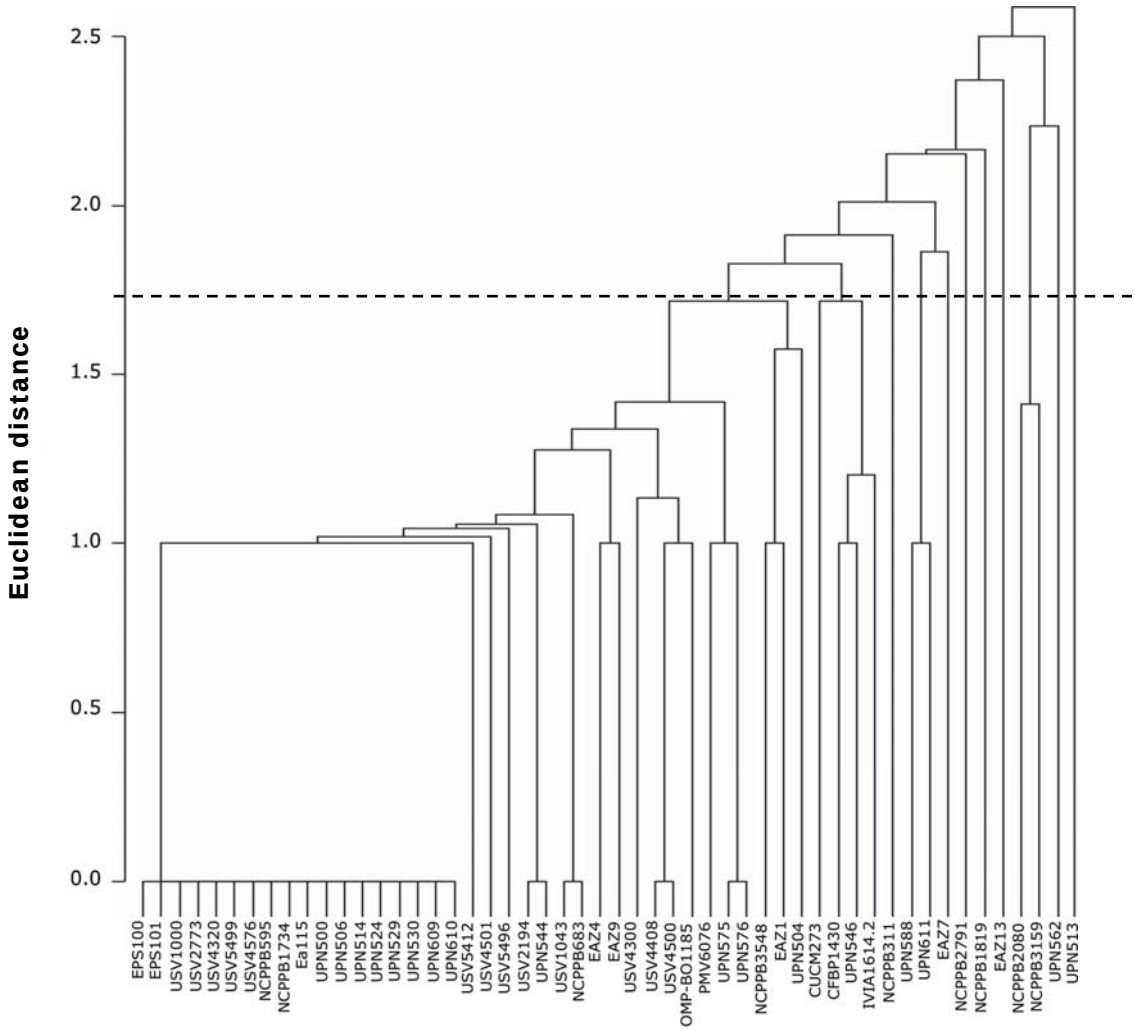

Figure 2.11. Dendrogram indicating the relatedness between the strains listed in *Table 2.2* as determined using Biolog GN system data. Euclidean distances were calculated on the basis of the differences between profiles. These data were then clustered using the agglomerative hierarchical method. Dotted line indicates the Euclidean distance to obtain the groups described in *Table 2.10.*

2.2. RFLPs analysis

2.2.1. RFLP fingerprinting of *ams* gene

Preliminary experiments were performed to optimise PCR conditions to get suitable amplification of *ams* gene without unspecific bands that lead to an additional step of purification before digestion. Previous assays without DNA extraction gave no signal with some reference strains (*Figure 2.12-A*). DNA extracted (*Figure 2.12-B*) from bacterial cultures using a commercial Kit, gave suitable amplifications and a PCR product of the expected length (~1600 bp).

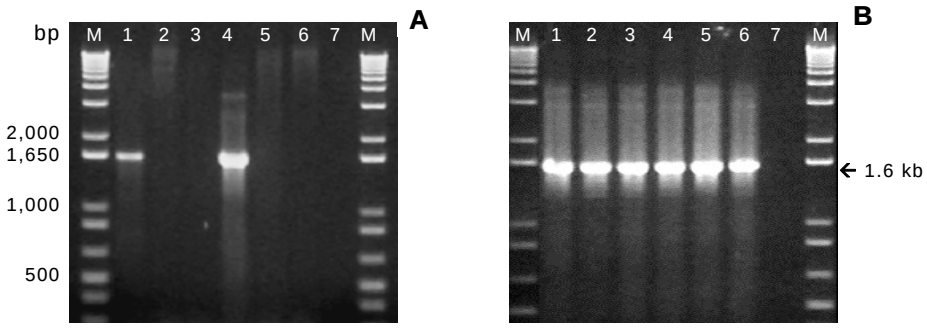

Figure 2.12. Amplification obtained using the *ams* gene primers (~1600 bp) without (**A**) and with DNA extraction (**B**). PCR products were separated by gel electrophoresis in a 1.5% agarose gel. Lanes 1 to 7, strains CFBP6076, USV4408, UPN500, USV1000, IVIA1614.2, EPS101 and negative control; M, 1 Kb plus DNA ladder.

The amplification products with the *ams* gene primer set of a reduced number of *E. amylovora* strains were digested using three restriction enzymes (*Rsa* I, *Mlu* I and *Msp* I). The digestion with *Mlu* I produced two fragments (998 and 637 bp). Digestion with *Rsa* I produced four fragments (39, 308, 298 and 990 bp). Digestion with *Msp* I produced 6 fragments (21, 70, 173, 299, 420 and 652 bp). *Figure 2.13* shows agarose gels in which fragments larger than 70 bp were separated.

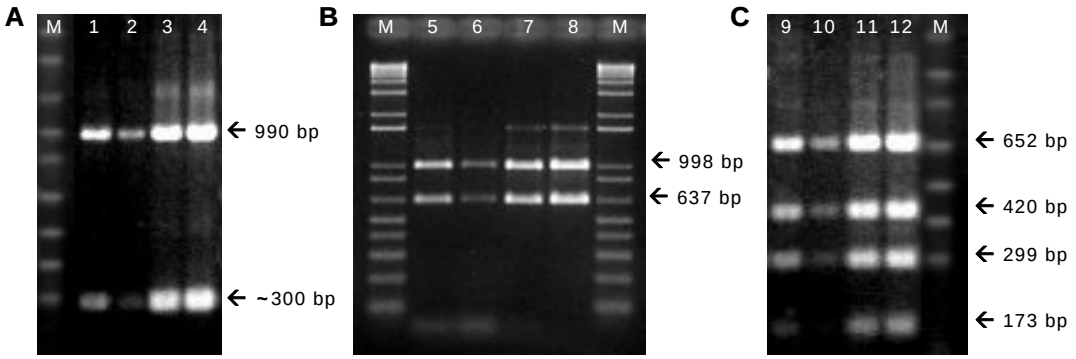

Figure 2.13. Agarose gels showing bands obtained after restriction analysis of *ams* gene of several *E. amylovora* strains. PCR products were digested with *Rsa* I (**A**), *Mlu* I (**B**), and *Msp* I (**C**), and separated by gel electrophoresis in a 2.5% agarose gel. Lanes 1 to 4, strains PMV6076, USV4408, EPS101 and USV1000; lanes 5 to 8; strains PMV6076, USV4408, EPS101 and USV1000; lanes 9 to 12, strains PMV6076, USV4408, EPS101 and USV1000; M, 100 bp DNA ladder.

Because of the higher number of fragments obtained with *Msp* I digestions, a selection of more strains was analyzed using *Msp* I restriction enzyme (*Figure 2.14*). There were no differences in RFLPs of *ams* among *E. amylovora* strains from different origin (plant host, country).

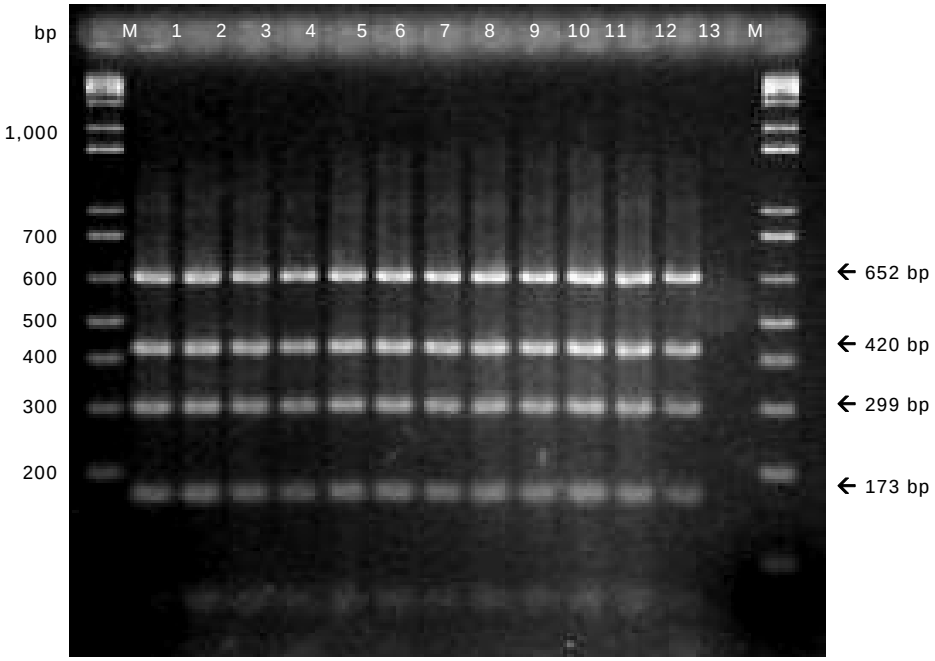

Figure 2.14. Agarose gel showing bands obtained after restriction analysis of *ams* gene digested with *Msp* I of 12 *E. amylovora* strains. PCR products were digested and separated by gel electrophoresis in a 2.5% agarose. Lanes 1 to 13, CFBP1430, NCPPB595, OMP-BO1185, CUCM273, EPS101, IVIA1614.2, USV1000, UPN605, UPN546, EAZ4, UPN529, CFBP6076, negative control; M, 100bp DNA ladder.

2.2.2. PFGE analysis of genomic DNA

Figure 2.15 shows PFGE profiles of genomic DNA from several *E. amylovora* strains after *Xba* I digestion. Strains UPN500, UPN546, UPN611, USV1000, EPS101 and EAZ4 showed the pattern *Pt4* independently of the region and host plant. Strain IVIA1614.2, isolated from *Crataegus* sp. in Segovia (Spain) showed pattern *Pt3* which was observed in strains like CFBP1430 from France and OMP-BO1185 from Italy. Strain PMV6076 did not present the same pattern than the parent strain CFBP1430. Among these strains two changes were observed: the 300 kb fragment was absent and a double band at 280 kb appeared. In addition, PMV6076 did not produce the 70 kb fragment. This strain presents a variation of pattern *Pt3*. Finally, pattern *Pt1* was observed in strain NCPPB595 isolated from *P. communis* in England and also in strain NCPPB3159 isolated from *Malus sylvestris* in Netherlands.

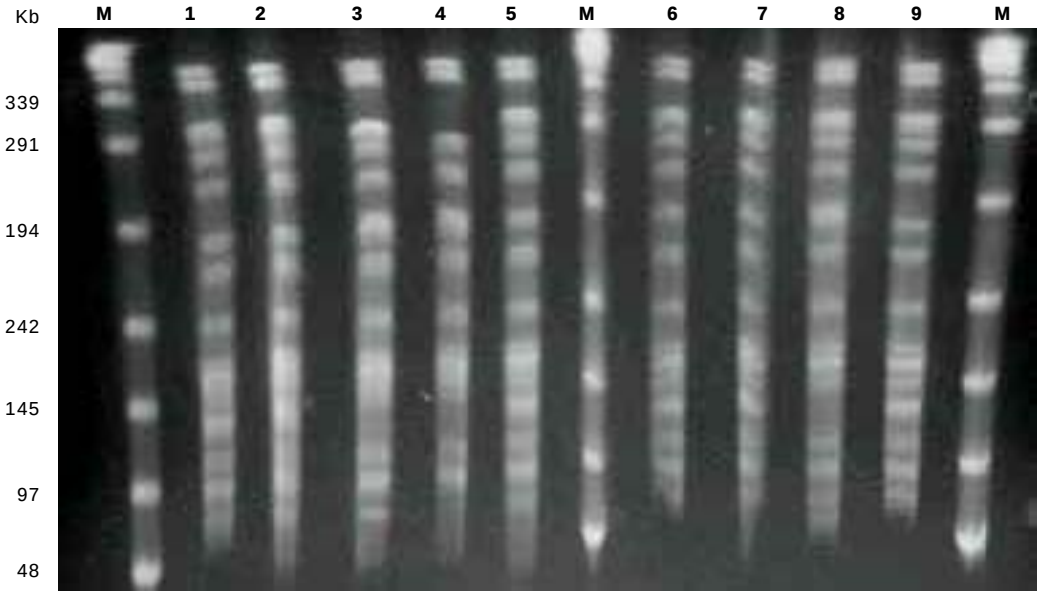

Figure 2.15. PFGE profiles of genomic DNA from various *E. amylovora* strains after *Xba* I digestion. The linear ramping time was 5 to 20s to 200 V. Lanes 1 to 9, strains EPS101, UPN500, CFBP1430, PMV6076, USV1000, NCPPB3159, EAZ4, IVIA1614.2, NCPPB595; M, Molecular weight ladders of phage λ DNA oligomers (Lambda ladder, New England Biolabs Inc., Beverly, MA, USA).

3. Virulence

3.1. Effect of *E. amylovora* concentration on infection of immature pear fruits

The incidence of infections in immature pear fruits at pathogen doses of 10^6 or 10^7 cfu·mL^{-1} was studied in 17 *E. amylovora* strains to determine suitable doses for the performance of the virulence assay.

A significant effect of the strain ($P<0.0001$, $F=139.46$), pathogen dose ($P<0.0001$, $F=160.19$) and interaction strain-dose ($P<0.0001$, $F=35.25$) was observed in the incidence of infections. The incidence of infections was greater at 10^7 cfu·mL^{-1}, though, for both doses, most of the strains developed levels of incidence around 100%. Only few strains developed significantly less incidence at 10^6 cfu·mL^{-1}.

Tukey's test showed that most of the strains did not differ significantly in the incidence of infections in immature pear fruits (*Table 2.11*), so we performed another assay to determine the optimal concentrations to observe sufficient differences among strains.

Figure 2.16 and *2.17* show the effect of the inoculation of *E. amylovora* at different concentrations in wounded immature pear fruits (cv. Passe Crassane). It can be observed that the relationship is described by a sigmoidal pattern.

Table 2.11. Incidence of infections on immature pear fruits by strains of *E. amylovora* when inoculated at two concentrations

	Pathogen concentration (cfu·mL^{-1})	
Strain[x]	**10^6**	**10^7**
UPN536	100 a	100 a
EPS101	100 a	100 a
NCPPB2080	100 a	100 a
NCPPB683	100 a	100 a
UPN506	100 a	100 a
UPN529	100 a	100 a
UPN544	100 a	100 a
UPN562	100 a	100 a
CUCM273	96 a	100 a
UPN546	96 a	100 a
NCPPB2791	88 ab	100 a
NCPPB1819	83 ab	100 a
UPN575	75 b	88 ab
UPN609	71 b	100 a
UPN611	21 c	83 b
UPN513	0 d	100 a
PMV6076	0 d	0 c

[x]Wounded immature pear fruits were inoculated with 10 µL of the pathogen suspension. Incidence was assessed after 7 days of incubation at 21 ºC and high relative humidity.
[y]Means within the same column followed by different letters are significantly different (P≤0.05) according to Tukey's test.

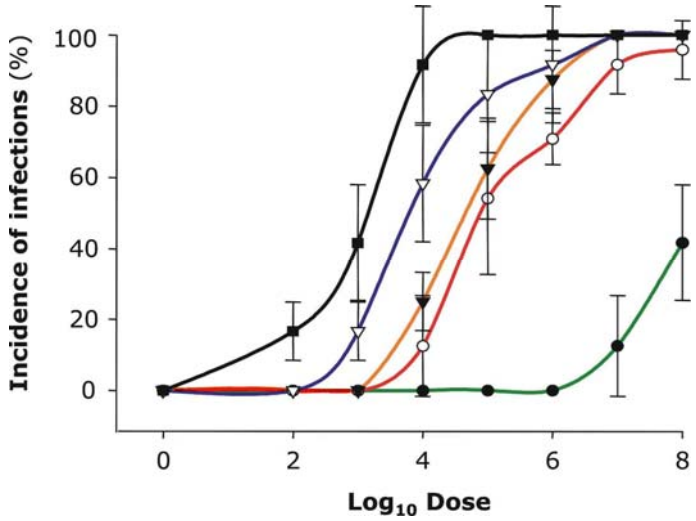

Figure 2.16. Infectivity titration of *E. amylovora* strain EPS101 on Passe Crassane immature fruits. Disease was assessed at 3 (●), 4 (○), 5 (▼), 7 (▽) and 10 (■) days after pathogen inoculation. Wounded immature pear fruits were inoculated with 10 µL of the pathogen suspension. Fruits were incubated at 21 ºC and high relative humidity.

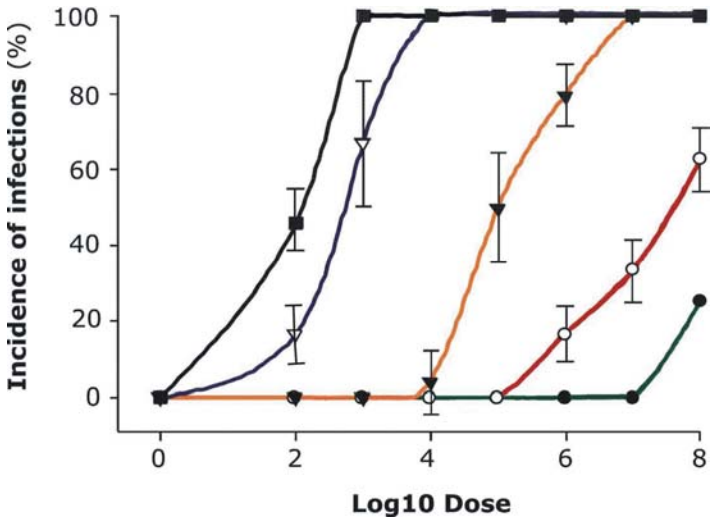

Figure 2.17. Infectivity titration of *E. amylovora* strain IVIA1614.2 on Passe Crassane immature fruits. Disease was assessed at 3 (●), 4 (○), 5 (▼), 7 (▽) and 10 (■) days after pathogen inoculation. Wounded immature pear fruits were inoculated with 10 µL of the pathogen suspension. Fruits were incubated at 21 ºC and high relative humidity.

The incidence after 5 days of inoculation was fit to pathogen dose using the probit and hyperbolic saturation functions and the resulting parameters are shown in *Table 2.12*. Both models adequately fit the data sets for 5 out of 6 strains studied on the basis of the goodness-of-fit obtained when comparing the observed data with the estimated data.

Table 2.12. Estimated parameters and goodness-of-fit of the probit and hyperbolic saturation models that relates the incidence of infections in immature pear fruits with the pathogen concentrations

		Probit				*Hyperbolic saturation*			
Strain	**Repetition**	λ ($\log_{10}ED_{50}$)	τ(*pathogen efficacy*)	**R^2**	**P>F**	Y_{max}	K_x (ED_{50})	**R^2**	**P>F**
EPS101	1	4.39	0.96	0.99	0.0000	0.96	19,024	0.99	0.0000
	2	4.17	0.99	0.99	0.0000	0.96	45,746	0.99	0.0000
	3	5.18	0.89	0.98	0.0000	0.96	121,216	0.98	0.0000
IVIA1614.2	1	5.00	0.86	0.99	0.0000	0.99	97,851	0.99	0.0000
	2	5.18	0.89	0.98	0.0000	0.96	121,216	0.98	0.0000
	3	5.18	0.89	0.98	0.0000	0.99	121,216	0.98	0.0000
NCPPB595	1	5.74	1.22	0.98	0.0000	0.93	199.791	0.93	0.0003
	2	6.00	0.86	0.99	0.0000	0.98	961,917	0.98	0.0000
	3	6.50	0.71	0.99	0.0000	0.98	961,917	0.98	0.0000
UPN529	1	5.08	1.25	0.95	0.0002	0.91	59,322	0.92	0.0006
	2	4.98	1.55	0.95	0.0002	0.84	29,484	0.96	0.0001
	3	4.77	1.43	0.99	0.0000	0.85	21,794	0.99	0.0000
UPN611	1	5.11	1.06	0.95	0.0002	0.96	102,110	0.95	0.0002
	2	4.63	0.55	0.98	0.0000	1.02	44,443	0.98	0.0000
	3	4.64	0.59	0.97	0.0000	0.94	38,065	0.98	0.0000

Data correspond to the infectivity titration on Passe Crassane immature fruits after 5 days from the pathogen inoculation.

Strain NCPPB311 was unable to produce infection until 10 days after its inoculation in immature pear fruits, with incidence levels of 25% when was inoculated at 10^8 cfu·mL^{-1}. The experimental data of this strain could not be used for parameter estimation. *Figure 2.18* shows fitted models to the experimental data for strains EPS101 and IVIA1614.2.

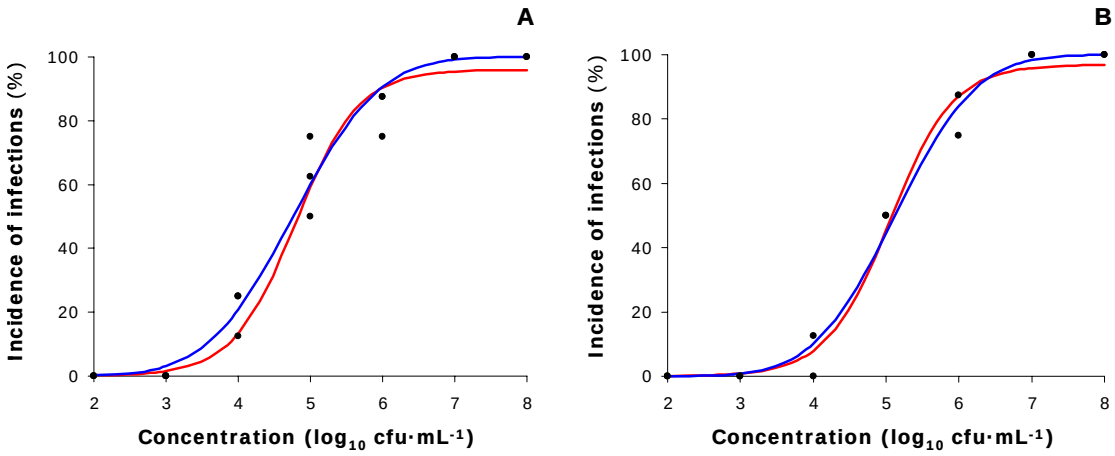

Figure 2.18. Effect of increasing concentration of *E. amylovora* EPS101 (**A**) and IVIA1614.2 (**B**) on development of infections in immature pear fruits. The lines represent the predictions according to the probit (blue line) and hyperbolic saturation (red line) models using the estimated parameters shown in *Table 2.9*.

Also models were fitted to data using a linear regression analysis. Thus, the probit model was fitted by transforming the incidence of infections to probit and the pathogen concentration to neperian logarithm (Ln). ED_{50} and *MID* were estimated from the equation obtained. *Table 2.13* shows the linear regression equation and ED_{50} and *MID* estimated by linear regression and goodness-of fit of lineal regression for each strain. It was noted that the fit obtained by means of the linear regression was not very good for some strains due to the few experimental points.

The classification of the strains by means of Tukey's test according to the ED_{50} and *MID* was quite similar between the different regression models (*Table 2.14*). Then, the parameters estimated by the probit model using non-linear regression were used to classify the strains.

ED_{50} for 4 out of 5 strains were similar (EPS101, IVIA1614.2, UPN529 and UPN611), around $6.0 \cdot 10^4$ cfu·mL^{-1}. However, strain NCPPB595 was less virulent with a value of $1.2 \cdot 10^6$ cfu·mL^{-1}. A higher variability was observed for *MID*, but only for the NCPPB595 strain was significantly lower than from the other *E. amylovora* strains ($F=10.33$, $P<0.0001$), with a value of $6.2 \cdot 10^4$ cfu·mL^{-1}. In contrast, strain UPN529 showed a value of $4.8 \cdot 10^2$ cfu·mL^{-1}.

Table 2.13. Linear regression equation obtained from linearized curves of incidence of infections with respect to pathogen concentration, and estimated ED_{50} and *MID* of several *E. amylovora* strains

Strain	Equation[x]	R^2	Prob > F	ED_{50} (cfu·mL^{-1})	*MID* (cfu·mL^{-1})
EPS101	y = 0.48 · x - 5.35	0.98	0.001	$7.17 \cdot 10^4$	$1.86 \cdot 10^3$
	y = 0.48 · x - 5.47	0.95	0.005	$9.20 \cdot 10^4$	$2.39 \cdot 10^3$
	y = 0.48 · x - 5.42	0.98	0.001	$8.16 \cdot 10^4$	$2.12 \cdot 10^3$
NCPPB595	y = 0.49 · x - 5.75	0.99	<0.001	$9.97 \cdot 10^4$	$3.01 \cdot 10^3$
	y = 0.48 · x - 5.45	0.98	0.001	$1.61 \cdot 10^5$	$4.19 \cdot 10^3$
	y = 0.48 · x - 5.74	0.97	0.002	$8.16 \cdot 10^4$	$2.98 \cdot 10^3$
IVIA1614.2	y = 0.63 · x - 6.34	0.96	0.007	$2.41 \cdot 10^4$	$1.49 \cdot 10^3$
	y = 0.63 · x - 6.34	0.96	0.007	$9.97 \cdot 10^4$	$3.01 \cdot 10^3$
	y = 0.49 · x - 5.75	0.99	<0.001	$2.41 \cdot 10^4$	$1.49 \cdot 10^3$
UPN595	y = 0.34 · x - 3.36	0.90	0.004	$1.93 \cdot 10^4$	$1.12 \cdot 10^2$
	y = 0.34 · x - 3.25	0.86	0.007	$1.59 \cdot 10^4$	$8.70 \cdot 10$
	y = 0.94 · x - 4.37	0.94	0.001	$1.24 \cdot 10^4$	$2.86 \cdot 10^3$
UPN611	y = 0.99 · x - 6.67	0.83	0.017	$7.96 \cdot 10^2$	$1.38 \cdot 10^2$
	y = 0.38 · x - 3.60	0.91	0.003	$1.40 \cdot 10^4$	$1.35 \cdot 10^2$
	y = 0.48 · x - 4.51	0.98	0.001	$1.20 \cdot 10^4$	$3.14 \cdot 10^2$

[x]Equation obtained by linear regression of the incidence of infections transformed to probit (y) and the pathogen concentration transformed to neparian logarithm (x).

Table 2.14. ED_{50} and *MID* from several *E. amylovora* strains calculated using different methods based on linear and non-linear regressions

	Non-linear regression				Linear regression	
	Probit model[x]		*Hyperbolic saturation model*		*Probit model*	
Strain	$\log_{10} ED_{50}$	$\log_{10} MID$	$\log_{10} ED_{50}$	$\log_{10} MID$	$\log_{10} ED_{50}$	$\log_{10} MID$
EPS101	4.76 a	3.10 a	4.67 a	3.31 a	4.91 b	3.32 ab
IVIA1614.2	4.79 a	3.58 ab	5.05 a	3.69 a	4.58 ab	3.27 b
NCPPB595	6.08 b	4.79 b	5.66 b	4.09 b	5.04 b	3.47 b
UPN529	4.94 a	2.69 a	4.63 a	3.21 a	4.19 a	2.48 ab
UPN611	4.79 a	3.46 ab	4.75 a	3.38 a	3.71 a	2.25 b

[x]Means within the same column followed by different letters are significantly different (P≤0.05) according to Tukey's test.
Estimated parameters from the experimental data obtained of dose-response experiments after 5 days of pathogen inoculation.

3.2. Kinetics of disease progression

Figure 2.20 shows disease progression curves for several *E. amylovora* strains at two concentrations. *K* and *AUDPC* were calculated directly from the disease progression curves, while r_g and t_0 were estimated by non-linear regression. Epidemiological models based on Gompertz, modified Gompertz, exponential, monomolecular and logistic functions were fit to experimental data (Data not shown). Only the modified Gompertz model provided adequate fit to experimental data (*Figure 2.20*), while the other models did not fit well, or the estimated parameters were not suitable to perform the classification of *E. amylovora* strains according to their virulence.

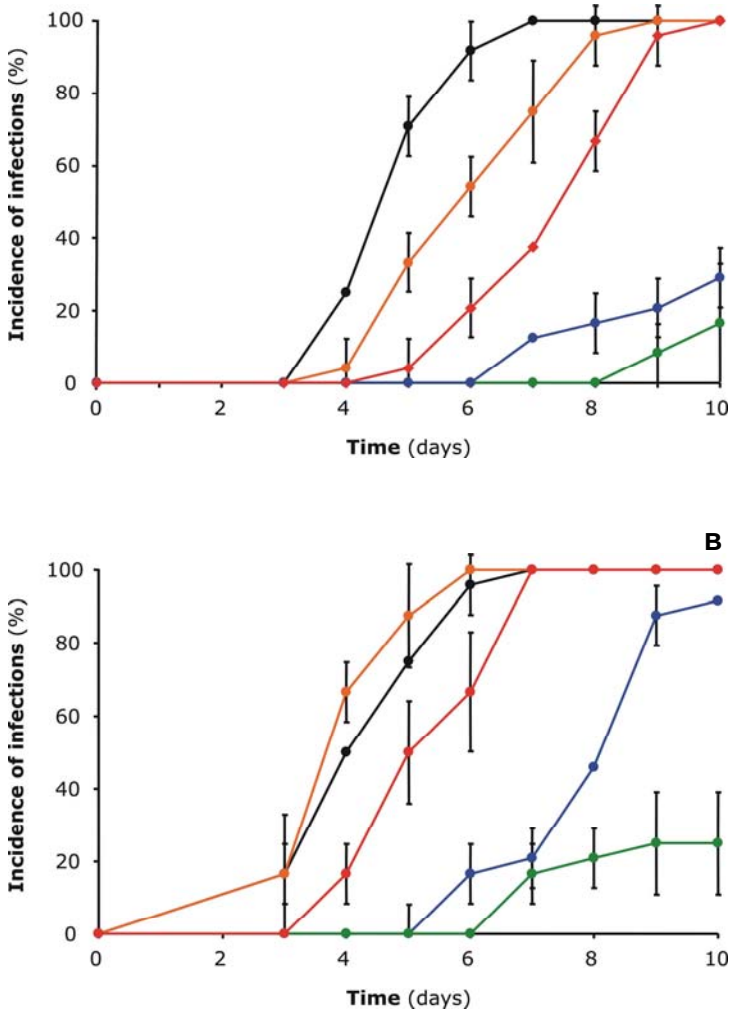

Figure 2.19. Disease progression in Passe Crassane immature fruits inoculated with several strains of *E. amylovora* at 10^3 cfu·mL^{-1} (**A**) or 10^5 cfu·mL^{-1} (**B**). Strains were UPN500 (black line), EPS101 (orange line), CFBP1430 (red line), NCPPB311 (blue line) and NCPPB595 (green line). Data points correspond to the mean incidence of three repetitions of six fruits per repetition, and bars indicate the confidence interval for the mean.

Estimation of parameters and goodness-of-fit for the modified Gompertz model for each strain are presented in *Annex 3*.

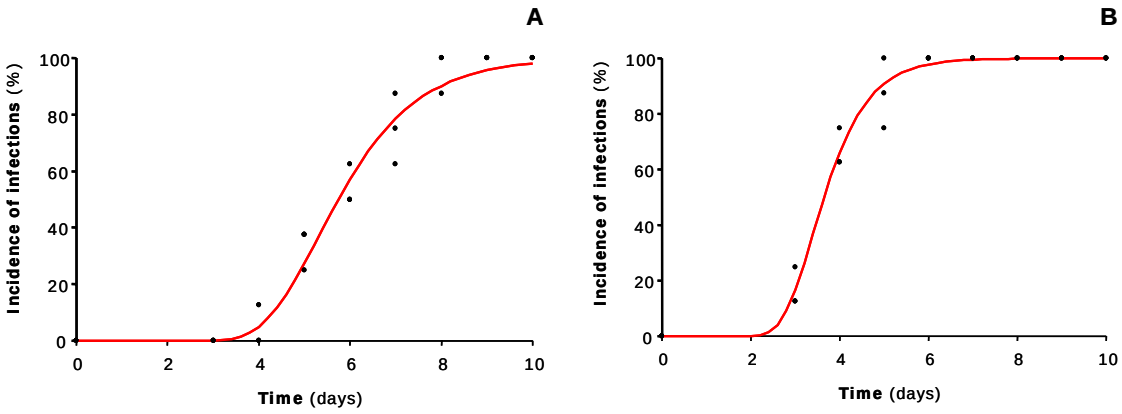

Figure 2.20. Disease progression curves in immature pear fruits inoculated with *E. amylovora* EPS101 at 10^3 (**A**) and at 10^5 cfu·mL^{-1} (**B**). The red line represents the predictions of disease progression curves according to the modified Gompertz model, using the estimated parameters shown in the *table 2.13*. Incidence of infections was assessed after 7 days from the pathogen inoculation.

A significant effect of dose, strain and the interaction strain-dose in the disease progression parameters (*K, AUDPC,* r_g and t_0) was observed (*Table 2.15*). Means separation of disease progression parameters for each of the two pathogen doses showed the existence of a slight gradient of virulence among *E. amylovora* strains (*Table 2.16* and *2.17*). Although, for the dose of 10^3 cfu·mL^{-1}, most of the strains were unable to infect immature pear fruits. For that reason the classification of strains was analyzed according to the results at 10^5 cfu·mL^{-1}.

Table 2.15. Effect of the strain of *E. amylovora* and dose of inoculation in Passe Crassane immature fruits on r_g, t_0, *K* and *AUDPC*

Parameter	Source[x]	DF	Mean Square	F value	P > F
r_g	Strain	52	1.26	51.37	< 0.0001
	Dose	1	10.96	446.71	< 0.0001
	Strain-dose	52	0.38	15.33	< 0.0001
	Error	212	0.02		
t_0	Strain	52	33.20	317.72	< 0.0001
	Dose	1	394.58	3,764.04	< 0.0001
	Strain-dose	52	5.66	54.04	< 0.0001
	Error	212	0.104		
I_{max}	Strain	52	22.52	240.81	< 0.0001
	Dose	1	221.57	2,369.58	< 0.0001
	Strain-dose	52	2.88	30.87	< 0.0001
	Error	212	0.09		
AUDPC	Strain	52	7,039.56	35.12	< 0.0001
	Dose	1	42,603.56	212.57	< 0.0001
	Strain-dose	52	1,663.44	8.15	< 0.0001
	Error	212	200.92		

[x]53 *E. amylovora* strains were inoculated at 10^3 or 10^5 cfu·mL^{-1} in Passe Crassane wounded immature fruits. The incidence of infections was assessed after 5 days of the pathogen inoculation.

Table 2.16. Disease progression parameters (r_g and t_o) for 53 *E. amylovora* strains according to the immature pear fruit assay. Parameters were estimated by non-linear regression using modified Gompertz model

	r_g			t_o	
Strain[x]	**High dose**	**Low dose**	**Strain**	**High dose**	**Low dose**
USV4501	1.73 a	0.91 hijk	NCPPB311	10.00 a	10.00 a
USV2773	1.70 ab	1.42 bcdef	PMV6076	10.00 a	10.00 a
USV1043	1.66 abc	0.64 jkl	UPN562	7.64 b	10.00 a
UPN536	1.65 abcd	1.06 fghij	UPN546	7.61 b	10.00 a
UPN504	1.64 abcd	1.19 cdefghi	UPN610	7.43 b	10.00 a
USV4300	1.61 abcde	1.46 bcdef	UPN609	7.25 b	10.00 a
USV4496	1.57 abcdef	0.50 klm	EAZ4	6.21 c	10.00 a
UPN513	1.56 abcdef	1.36 bcdefg	NCPPB595	6.01 cd	7.95 c
UPN500	1.56 abcdef	1.52 bcd	NCPPB2791	5.88 cd	10.00 a
USV4320	1.54 abcdefg	1.59 abc	EPS100	5.62 cde	6.00 d
UPN544	1.54 abcdefg	0.80 hijk	USV2194	5.34 cdef	8.72 b
USV4512	1.53 abcdefg	1.07 efghij	UPN576	5.28 cdef	4.15 efghi
UPN529	1.51 abcdefg	0.60 klm	UPN588	5.07 defg	7.48 c
USV4576	1.49 abcdefg	1.51 bcde	EAZ9	4.79 efg	10.00 a
EPS101	1.46 abcdefg	0.84 hijk	EAZ13	4.72 efg	10.00 a
USV4499	1.41 abcdefgh	1.16 cdefghi	UPN575	4.69 efg	10.00 a
NCPPB2791	1.40 abcdefgh	0.00 n	NCPPB3548	4.66 efg	10.00 a
UPN576	1.37 abcdefghi	0.54 klm	NCPPB1819	4.40 fgh	5.81 d
UPN588	1.36 abcdefghij	0.28 lmn	EAZ7	4.17 ghi	10.00 a
UPN514	1.35 abcdefghijk	1.15 cdefghi	UPN536	3.59 hij	3.79 efghijklm
USV4408	1.33 abcdefghijk	1.09 defghij	NCPPB3159	3.21 ijk	4.39 ef
UPN611	1.23 abcdefghijkl	1.25 cdefgh	NCPPB683	3.14 ijkl	3.67 efghijklm
EAZ4	1.16 bcdefghijkl	0.00 n	UPN524	3.13 ijkl	10.00
UPN506	1.13 cdefghijkl	0.00 n	USV4320	3.07 jklm	4.21 efghi
USV4500	1.13 cdefghijkl	0.74 ijk	USV4300	3.06 jklm	4.00 efghijkl
CUCM273	1.13 cdefghijkl	1.09 defghij	NCPPB2080	3.03 jklm	5.29 d
NCPPB2080	1.12 cdefghijkl	0.85 hijk	UPN530	2.97 jklmn	4.11 efghij
USV1000	1.12 cdefghijkl	0.55 klm	UPN500	2.87 jklmno	4.31 efg
NCPPB3159	1.11 cdefghijkl	0.81 hijk	UPN506	2.86 jklmno	10.00 abcde
EAZ9	1.09 defghijkl	0.00 n	USV2773	2.86 jklmno	4.35 ef
NCPPB595	1.05 efghijkl	0.59 klm	USV4499	2.84 jklmno	3.41 ghijklmn
CFBP1430	1.05 efghijkl	0.85 hijk	USV4501	2.84 jklmno	3.39 hijklmn
IVIA1614.2	1.03 fghijkl	0.91 hijk	USV4496	2.79 jklmno	5.41 d
NCPPB3548	0.99 ghijkl	0.00 n	UPN504	2.75 jklmno	3.53 fghijklm
UPN530	0.99 ghijkl	0.94 ghijk	USV1000	2.73 jklmno	3.23 jklmno
NCPPB683	0.88 hijklm	0.86 hijk	UPN529	2.69 jklmnop	4.02 efghijkl
UPN524	0.88 hijklm	0.00 n	NCPPB1734	2.37 klmnopq	4.19 efghi
NCPPB1734	0.85 ijklm	0.95 ghijk	Ea115	2.29 klmnopq	10.00 a
EAZ1	0.83 ijklm	0.91 hijk	UPN514	2.21 klmnopq	2.38 o
NCPPB1819	0.82 jklm	0.81 hijk	USV4500	2.13 klmnopq	4.38 ef
UPN575	0.81 jklm	0.00 n	UPN611	2.06 klmnopq	3.17 lmno
EAZ7	0.81 klm	0.00 n	USV4408	2.04 lmnopq	4.07 efghijk
EAZ13	0.76 lm	0.00 n	IVIA1614.2	1.99 lmnopq	3.04 mno
OMP-BO1185	0.72 lmn	0.55 klm	CUCM273	1.96 mnopq	4.10 efghij
Ea115	0.69 lmn	0.00 n	UPN544	1.94 mnopq	4.29 efgh
USV2194	0.44 mno	0.85 hijk	USV1043	1.92 mnopq	4.26 efgh
UPN609	0.42 mno	0.00 n	EPS101	1.86 opq	3.51 fghijklm
UPN610	0.40 mno	0.00 n	USV4512	1.78 opq	2.54 no
UPN562	0.39 mno	0.00 n	EAZ1	1.74 opq	4.51 e
UPN546	0.38 mno	0.00 n	USV4576	1.74 opq	2.92 mno
EPS100	0.24 no	0.18 mn	UPN513	1.58 pq	3.19 klmno
NCPPB311	0.00 o	0.00 n	OMP-BO1185	1.54 q	3.34 ijklmn
PMV6076	0.00 o	0.00 n	CFBP1430	1.29 q	2.61 no

[x]Means within the same column followed by different letters are significantly different (P≤0.05) according to Ryan-Einot-Gabriel-Welsh Multiple Range test.

Experimental data correspond the incidence of infections after 5 days of inoculation at two pathogen densities, 10^3 (low dose) and 10^5 (high dose) cfu·mL^{-1}.

Color gradation indicates degree of virulence; dark gray, high virulence; soft gray, moderate virulence; pale gray, low virulence.

Table 2.17. Disease progression parameters (*K* and *AUDPC*) for 53 *E. amylovora* strains according to the immature pear fruit assay. *K* was estimated by non-linear regression using modified Gompertz model, whereas AUDPC was calculated directly from data

	K			*AUDPC*	
Strain[x]	**High dose**	**Low dose**	**Strain**	**High dose**	**Low dose**
USV4496	100 a	38 defg	UPN513	6.96 a	5.00 abcd
USV4499	100 a	100 a	USV1043	6.67 ab	2.36 h
USV4500	100 a	67 abcd	USV4512	6.54 abc	5.63 a
USV4501	100 a	92 ab	USV4576	6.54 abc	5.21 abcd
USV4512	100 a	100 a	EPS101	6.38 abcd	4.13 fg
USV4576	100 a	100 a	UPN544	6.38 abcd	4.00 fg
CUCM273	100 a	92 ab	USV4408	6.13 abcde	4.63 cdefg
UPN500	100 a	100 a	CUCM273	6.09 abcde	4.05 fg
UPN504	100 a	100 a	CFBP1430	6.09 abcde	5.38 abc
NCPPB683	100 a	100 a	OMP-BO1185	6.05 abcdef	4.75 bcdef
EPS101	100 a	96 a	USV4320	5.88 bcdefg	4.34 efg
EAZ1	100 a	100 a	USV4501	5.88 bcdefg	4.13 fg
EAZ4	100 a	88 abc	USV4500	5.84 bcdefg	2.89 h
NCPPB2080	100 a	67 abcd	UPN514	5.79 bcdefg	5.46 ab
UPN530	100 a	100 a	EAZ1	5.67 bcdefg	4.04 fg
USV1043	100 a	46 cdefg	UPN611	5.59 bcdefg	4.75 cbdef
UPN536	100 a	88 abc	USV2773	5.54 cdefg	4.09 fg
CFBP1430	100 a	100 a	UPN506	5.50 cdefg	0.00 k
USV4300	100 a	100 a	UPN529	5.42 defgh	1.19 ij
OMP-BO1185	100 a	100 a	USV4496	5.34 defgh	1.36 i
USV1000	100 a	92 ab	USV4300	5.17 efghi	4.55 defg
UPN529	100 a	21 efg	IVIA1614.2	5.17 efghi	4.59 defg
UPN513	100 a	100 a	UPN504	5.17 efghi	4.67 cdefg
UPN514	100 a	100 a	UPN500	5.17 efghi	4.21 efg
USV4408	100 a	100 a	USV4499	5.13 efghi	4.50 defg
UPN506	100 a	0 g	USV1000	4.96 fghij	4.21 efg
USV4320	100 a	100 a	NCPPB2080	4.91 ghij	2.79 h
UPN544	100 a	67 abcd	NCPPB1734	4.42 hijk	3.84 g
UPN611	100 a	100 a	NCPPB683	4.42 hijk	3.88 g
USV2773	100 a	100 a	UPN524	4.37 hijk	0.00 k
IVIA1614.2	100 a	100 a	NCPPB3159	4.25 ijkl	4.00 fg
UPN524	96 a	0 g	UPN530	4.21 ijklm	3.88 g
UPN576	92 ab	58 abcde	UPN536	4.21 ijklm	4.00 fg
NCPPB1734	88 abc	88 abc	USV2194	4.04 jklmn	0.00 k
UPN588	88 abc	13 fg	Ea115	3.67 klmno	0.00 k
NCPPB2791	88 abc	0 g	EAZ7	3.67 klmno	0.00 k
EAZ9	88 abc	0 g	UPN576	3.37 klmno	2.33 h
Ea115	79 abcd	13 fg	UPN588	3.29 lmno	0.56 jk
NCPPB3548	75 abcde	0 g	NCPPB3548	3.17 mno	0.00 k
EAZ13	71 abcdef	17 efg	EAZ9	3.13 no	0.00 k
NCPPB1819	67 abcdef	59 abcde	NCPPB1819	2.75 op	2.19 h
EAZ7	58 abcdef	0 g	EAZ13	2.63 op	0.00 k
NCPPB3159	50 bcdef	50 bcdef	EAZ4	2.63 op	0.00 k
NCPPB595	46 cdefg	0 g	NCPPB595	1.97 pq	0.27 k
UPN575	33 efgh	0 g	UPN575	1.39 qr	0.00 k
USV2194	29 fgh	4 g	USV2194	1.21 qrs	0.28 k
UPN562	29 fgh	0 g	UPN562	0.84 rst	0.00 k
EPS100	29 fgh	21 efg	EPS100	0.57 rst	0.56 jk
UPN546	4 gh	0 g	UPN610	0.39 rst	0.00 k
UPN609	4 gh	0 g	UPN609	0.39 rst	0.00 k
UPN610	4 gh	0 g	UPN546	0.24 st	0.00 k
PMV6076	0 h	0 g	NCPPB311	0.00 t	0.00 k
NCPPB311	0 h	0 g	PMV6076	0.00 t	0.00 k

[x]Means within the same column followed by different letters are significantly different (P≤0.05) according to Ryan-Einot-Gabriel-Welsh Multiple Range test.

Experimental data correspond the incidence of infections after 5 days of inoculation at two pathogen densities, 10^3 (low dose) and 10^5 (high dose) cfu·mL^{-1}.

Color gradation indicates degree of virulence; dark gray, high virulence; soft gray, moderate virulence; pale gray, low virulence.

Table 2.18 shows strains classified on the basis of each disease progression parameter at 10^5 cfu·mL^{-1} by means of a subjective grouping according to the arrangement obtained by the means separation test for each parameter (*K, AUDPC,* r_g and t_0). Strains were clustered in three groups corresponding to high, moderate and low virulence. For all parameters the classification in virulence groups was similar, except for *K*, where most of strains presented high virulence according to *K*.

It was noted that few strains were classified in the same level of virulence independently of the parameter used. Thus, 6 strains presented high virulence (EPS101, UPN513, UPN544, USV1043, USV4512 and USV4576) for the four parameters, 8 low virulence (EPS100, NCPPB311, PMV6076, UPN546, UPN562, UPN575, UPN609 and UPN611) and only 1 moderate virulence (NCPPB3159). The remaining strains did not present consistent results and the classification depends on the parameter used. For example, strains EAZ4 and OMP-BO1185 presented low virulence according to r_g, while high virulence for other three parameters.

Table 2.18. Clustering of *E. amylovora* strains in virulence groups (high, moderate and low virulent) according to the virulence parameters (r_g, t_0, *K* and *AUDPC*) estimated from the disease progression curves in immature pear fruits when inoculated at 10^5 cfu·mL^{-1}

Parameter	**Virulence groups**		
	High	*Moderate*	*Low*
r_g	EPS101, UPN500, UPN504, UPN513, UPN529, UPN536, UPN544, USV1043, USV2773, USV4300, USV4320, USV4496, USV4501, USV4512, USV4576	EAZ4, EAZ9, CFBP1430, CUCM273, IVIA1614.2, NCPPB595, NCPPB2080, NCPPB2791, NCPPB3159, NCPPB3548, UPN506, UPN514, UPN530, UPN576, UPN588, UPN611, USV1000, USV4408, USV4499, USV4500	Ea115, EAZ1, EAZ7, EAZ13, EPS100, NCPPB311, NCPPB683, NCPPB1734, NCPPB1819, OMP-BO1185, PMV6076, UPN524, UPN546, UPN562, UPN575, USV2194, UPN609, UPN610
t_0	EAZ1, EPS101, CFBP1430, CUCM273, IVIA1614.2, OMP-BO1185, UPN513, UPN544, UPN611, USV1043, USV4408, USV4512, USV4576	Ea115, NCPPB683, NCPPB1734, NCPPB2080, NCPPB3159, UPN500, UPN504, UPN506, UPN514, UPN524, UPN529, UPN530, UPN536, USV1000, USV2773, USV4300, USV4320, USV4496, USV4499, USV4500, USV4501	EAZ4, EAZ7, EAZ9, EAZ13, EPS100, NCPPB311, NCPPB595, NCPPB1819, NCPPB2791, NCPPB3548, PMV6076, UPN546, UPN562, UPN575, UPN576, UPN588, UPN609, UPN610, USV2194
K	EAZ1, EAZ4, EPS101, CFBP1430, CUCM273, IVIA1614.2, NCPPB683, NCPPB2080, OMP-BO1185, UPN500, UPN504, UPN506, UPN513, UPN514, UPN524, UPN529, UPN530, UPN536, UPN544, UPN576, UPN611, USV1000, USV1043, USV2773, USV4408, USV4300, USV4320, USV4496, USV4499, USV4500, USV4501, USV4512, USV4576	EAZ7, EAZ9, Ea115, EAZ13, NCPPB595, NCPPB1734, NCPPB1819, NCPPB2791, NCPPB3159, NCPPB3548, UPN588	EPS100, PMV6076, NCPPB311, UPN546, UPN562, UPN575, UPN609, UPN610, USV2194
AUDPC	EAZ1, EPS101, CFBP1430, CUCM273, OMP-BO1185, UPN506, UPN513, UPN514, UPN544, UPN611, USV1043, USV2773, USV4408, USV4320, USV4500, USV4501, USV4512, USV4576	IVIA1614.2, NCPPB683, NCPPB1734, NCPPB2080, NCPPB3159, UPN500, UPN504, UPN524, UPN529, UPN530, UPN536, USV1000, USV2194, USV4300, USV4496, USV4499	Ea115, EAZ4, EAZ7, EAZ9, EAZ13, EPS100, PMV6076, UPN546, UPN562, UPN575, NCPPB311, NCPPB595, NCPPB1819, NCPPB3548, UPN576, UPN588, UPN609, UPN610, USV2194

Gray, green and red marks correspond to the strains classified as high, moderate and low virulent for all the parameters.

In most cases, the virulence of strains decreased drastically when inoculated at 10^4 cfu·mL^{-1}, and some strains were unable to infect immature pear fruits. Nevertheless, some strains maintained the same level of virulence for both doses, like strains USV2773, USV4512, USV4576 and UPN513 with high virulence at both tested doses. Other strains like USV4501, USV1043, UPN544 and EPS101 only presented high virulence at 10^6 cfu·mL^{-1}, but they lost virulence at 10^3 cfu·mL^{-1}. The most aggressive strain was USV4320 with high r_g at both concentrations, while the most infective strain was CFBP1430 with the lowest t_0 at both concentrations.

When strains were classified according the *CVI* (*Table 2.19*), the classification was similar to the obtained by *AUDPC,* r_g and t_0. Thus 13 strains presented high virulence (25%), 19 moderate virulence (36%) and 21 low virulence (40%). The most virulent strains were EPS101, UPN513, UPN544, USV1043, USV4512 and USV4576. While the less virulent strains were EPS100, NCPPB311, PMV6076, UPN546, UPN562, UPN575, UPN609, UPN610 and UPN610.

Table 2.19. Strains clustered in high, moderate and low virulence according to Composite Virulence Index (*CVI*)

Virulence group	**Strains**
High (*CVI* > 0.9)	EPS101 (1.0), UPN513 (1.0), UPN544 (1.0), USV1043 (1.0), USV4512 (1.0), USV4576 (1.0), CFBP1430 (0.92), CUCM273 (0.92), UPN611 (0.92), USV2773 (0.92), USV4320 (0.92), USV4408 (0.92), USV4501 (0.92)
Moderate (0.9 ≤ *CVI* > 0.6)	EAZ1 (0.83), IVIA1614.2 (0.83), UPN500 (0.83), UPN504 (0.83), UPN514 (0.83), UPN529 (0.83), UPN536 (0.83), USV4300 (0.83), USV4496 (0.83), USV4500 (0.83), NCPPB2080 (0.75), UPN524 (0.75), USV4499 (0.75), OMP-BO1185 (0.75), UPN530 (0.75), USV1000 (0.75), NCPPB683 (0.67), NCPPB3159 (0.67), UPN506 (0.67)
Low or non-virulent (*CVI* ≤ 0.6)	EAZ4 (0.58), NCPPB1734 (0.58), UPN576 (0.58), Ea115 (0.50), EAZ9 (0.50), NCPPB595 (0.50), NCPPB2791 (0.50), UPN588 (0.50), NCPPB3548 (0.50), EAZ7 (0.42), EAZ13 (0.42), NCPPB1819 (0.42), USV2194 (0.42), EPS100 (0.33), NCPPB311 (0.33), PMV6076 (0.33), UPN546 (0.33), UPN562 (0.33), UPN575 (0.33), UPN609 (0.33), UPN610 (0.33)

The figures presented between brackets corresponded to the *CVI* of each strain.

4. Optimization of model systems for interaction studies

4.1. Pear blossom model

Two inoculation methods were evaluated in pear flowers. The methods consisted of the deposition of a drop of 10 μL suspensions in pistil and pistil rubbing with a paintbrush soaked in the pathogen suspension. Two pathogen concentrations (10^7 and 10^8 cfu·mL^{-1}) were used. Significant differences were not observed neither in the incidence nor in the severity between the inoculation methods. Although, significant differences, in the severity of infections were observed between the concentrations of pathogen ($P<0.001$), obtaining a greater severity when the pathogen was inoculated at the high dose. The pathogen developed severity and incidence levels around 80% when was inoculated at 10^7 cfu·mL^{-1} and around 100% when was inoculated at 10^8 cfu·mL^{-1} (*Table 2.20)*.

Table 2.20. Effect of the inoculation method and pathogen concentration on the incidence and severity of *E. amylovora* infections in pear flowers (cv. Doyenne du Comice)

Inoculation method	**Dose (cfu·mL^{-1})**	**Incidence** (%)^x	**Severity** (%)
Drop of 10 μL	10^8	100 a	100 a
	10^7	76 b	80 b
Soaked paintbrush	10^8	100 a	100 a
	10^7	83 b	88 b

Means from the same column and inoculation method with different letter are significantly different ($P\leq0.05$) according to Tukey's test.

4.2. Young pear shoot model

In the experiment performed to establish suitable pathogen concentrations, significant differences were observed in severity and incidence between pathogen concentrations at 10^6 cfu·mL^{-1} and 10^7 cfu·mL^{-1} ($P<0.001$) (*Table 2.21*). Significant differences were also observed between the severity (40%) and incidence (62%) of *E. amylovora* infections when the pathogen was inoculated at 10^6 cfu·mL^{-1}. Thus, the suitable concentration of pathogen selected was of 10^7 cfu·mL^{-1}.

Table 2.21. Effect of the *E. amylovora* EPS101 concentration on the incidence and severity of infections in young shoots (cv. Doyenne du Comice)

EPS101 Concentration (cfu·mL^{-1})	**Incidence** (%)^x	**Severity** (%)
10^7	86 a	79 a
10^6	62 b	40 b

Means in the same column with different letter are significantly different ($P\leq0.05$) according to Tukey's test.

DISCUSSION

This chapter is focused on the selection of a group of strains of *E. amylovora* to carry out the screening of potential biocontrol agents of fire blight, which is the main object of the present PhD thesis. The Chapter deals also with the optimization of laboratory pathosystem models based on apple/pear blossoms and shoots to be used for the selection of potential biocontrol agents of fire blight and in studies of interaction between host, pathogen and biocontrol agents.

The first step proposed for the development of this study was to build-up a collection of strains of *E. amylovora*. A screening was done in our influence region. In spite of some symptomatic samples from pear and apple commercial orchards and from nurseries of ornamental plants were processed during the study, *E. amylovora* was not isolated. This was because most of samples proceeded from the Girona province, a zone where fire blight is not yet present. *E. amylovora* was only isolated from a sample obtained from a commercial pear orchard of Lleida where fire blight was detected in 1998 (Lopez *et al.,* 1999).

Because it was impossible to collect enough strains from our survey to complete the collection, strains from other sources including isolates from Catalunya and Spain and from other countries were included to increase the representativeness. The collection was finally composed of 53 strains with 74% of strains isolated in Spain and the rest from other countries like England, Egypt, France, USA, Italy, Turkey, New Zealand, the Netherlands and Bulgaria.

Early evidence of strain diversity and its importance in the understanding of epidemiology of fire blight and detection has been summarized (Momol and Aldwinckle, 2000). Strains vary in virulence, colony morphology, serology, phage typing and sensitivity to antibiotics (Paulin, 2000). Several assays have been developed for detection of this pathogen to solve the lack of a definitive technique for its identification (Brennan *et al*., 2002). These methods range from the observation of disease symptoms and isolation and growth of the pathogen on semi-selective media (Miller and Schroth, 1972; Ishimaru and Klos, 1984), to serological assays (Lin *et al*., 1987; Gorris *et al*., 1996), and molecular techniques including plasmid-specific PCR analysis (Bereswill *et al*., 1992; Merighi, 1999), chromosome-specific PCR analysis (Bereswill *et al*., 1995), ribotyping (Brown *et al*., 1999), and random amplified polymorphic DNA (RAPD) analysis (Momol *et al*., 1997).

Some of these techniques such as PCR allow a more rapid identification than traditional techniques (Bereswill *et al.*, 1992), but as pointed out by other researchers, in spite of being a very useful and sensitive technique, it is still seriously limited due to inhibition by different compounds or to *E. amylovor*a strains diversity. In fact, the experience in diagnosing fire blight has shown the importance of this problem, sometimes detecting fewer positive samples by the standard PCR technique than by plating or enrichment-ELISA (Llop *et al*., 2000). This fact difficult the detection of *E. amylovora* in field samples, and therefore diagnosis of *E. amylovora* needs the combination of several methodologies from typical

symptoms, growth on different media to detection by means of molecular techniques. Moreover, it is advisable to contrast the results obtained by the conventional PCR (Bereswill *et al*., 1992) and enrichment DASI-ELISA (Gorris *et al*., 1996), with other complementary tests like the restriction of PCR products from pEA29 primers, detection by PCR using other primers described for *E. amylovora* targeted on *ams* gene involved in the biosynthesis of amylovoran (Bereswill *et al*., 1995), and *23S rDNA* gene (Maes *et al*., 1996) or detection by nested-PCR (McManus and Jones, 1995; Llop *et al*., 2000). Finally, it is also necessary to confirm the pathogenicity of suspected isolates on sensible material, like immature fruits or flowers.

Strains of *E. amylovora* from the collection performed in the present study were characterized at various levels including growth on different media, metabolic profile determined by API 20E and Biolog GN systems, amplification by PCR of pEA29 plasmid and *ams* gene, enrichment DASI-ELISA, intensity of infections in immature pear fruits, and RFLPs fingerprinting of *ams* gene and of genomic DNA. The characterization has shown that 4% of strains did not present domed circular mucoid colonies in SNA, 9% were not detected by enrichment DASI-ELISA, 7.5% were not detected by PCR using pEA29 primers, and 9% presented a low or inexistent pathogenicity in immature pear fruits. This indicates the existence of a very low heterogeneity among *E. amylovora* strains at cultural, serological and genetic level in accordance with several studies (Paulin and Samson, 1973; Vantomme *et al.,* 1982; McManus and Jones, 1995; Beer *et al*., 1996; Kim *et al*., 1996; Momol *et al*., 1997).

Even though, a high homogeneity among *E. amylovora* strains was observed between cultural characteristics and detection methods, greater variability was observed in the results of API and Biolog systems. In API system, few differences were observed among the groups defined. Differences among patterns were only around one or two characteristics. For Biolog GN system, few differences were also observed between groups in accordance with previous results of utilization of carbohydrates and organic acids. In this case the grouping was related in part to the host plant. Thus, the main group of strains clustered in a group mainly composed of strains isolated from pome fruit trees, while the rest of the groups included most strains isolated from ornamental plants. These results are in accordance with the study of Kim *et al*. (1996) based on carbon utilization using the Biolog system. In this study distinct groups of *E. amylovora* were defined according to the carbon sources utilization profile. Thus, strains isolated from *Maloideae* hosts clustered together. At least two different groups were recognized for strains pathogenic on *Rubus* sp. These results are in accordance with a certain host species-specificity described for some strains by other authors (Norelli *et al*., 1984; Beer *et al*., 1996).

A higher degree of variability was observed in virulence which permits strains to be classified by their virulence in three levels (high, moderate and low) using the immature pear fruit test. These results contrast with other studies where no significant differences were observed in the degree of virulence of several *E. amylovora* strains (Quamme and Bonn, 1981; Manulis *et al*., 1998), while are in concordance with others that describe differences in virulence (Shaffer and Goodman, 1962; Goodman 1973; Norelli *et al.*, 1984; Norelli *et al*., 1988;

Paulin *et al*., 1993). The ambiguity in the various reports might result from the different methods of assay of pathogenicity used in each study (Manulis *et al*., 1998).

Genomic diversity of various *E. amylovora* strains was assessed by RFLP analysis of *ams* gene and genomic MRFLP analysis by PFGE. The restriction patterns obtained on the RFLP analysis of *ams* gene were very simple and indistinguishable among strains. These results agree with the results obtained by Manulis *et al.* (1998) that did not found important differences among 205 strains from different origins, attending to the genomic diversity by RAPD analysis using several arbitrary primers, by DNA-DNA hybridization (Gardner and Kado, 1972), and by physiological and serological methods (Vantomme *et al*., 1982). In contrast, other studies using molecular techniques capable of detecting higher genetic variability allow grouping strains from different origins. Thus, repetitive DNA element and ribotyping PCR (McManus and Jones, 1995), RAPD fragment analysis (Momol *et al*., 1997) and ARDREA (Momol *et al*., 1999) allowed to distinguish strains isolated from fruit trees (Maloideae) from strains isolated from *Rubus* sp. Also new techniques that produce highly polymorphic markers, like inverse sequence-tagged repeat (ISTR) (Rico, 2003) analysis or the combination of AFLP analysis (Rico *et al.*, 2004) were successfully applied to reveal polymorphisms among Spanish isolates of *E. amylovora*, though the resulting clusters were not correlated with host, year or country of isolation.

Due to the limited genetic variability obtained by RFLP analysis of *ams* gene, the genomic MRFLP was used to analyze the genetic diversity among *E. amylovora* strains in the present work. The MRFLPs obtained by DNA digestion with *Xba* I were used to group strains according to five pattern types (Zhang and Geider, 1997; Zhang *et al.,* 1998). Despite, this technique described a higher genetic variability among strains than the RFLP analysis of *ams* gene, only three different PFGE patterns were observed among strains analyzed in the present study. This low diversity using the PFGE patterns appear to be clonally related with minor variations in PFGE profiles which seems to be related with their geographical origin (Zhang and Geider, 1997; Jock *et al*., 2002). Spanish strains (UPN500, UPN546, UPN611, USV1000, EPS101 and EAZ4) and strain NCPPB3159, isolated from Netherlands, presented the *pt4*. France and Italy strains and the Spanish strain IVIA1614.2, isolated in Segovia, presented the *pt3*. Finally, the strain from England NCPPB595 presented the *pt1*. Geider *et al.* (2000) have concluded that differentiation of *E. amylovora* strains by PFGE allow to determine the origin of *E. amylovora* in accordance with other studies (Zhang and Geider, 1997; Zhang *et al.,* 1998; Jock *et al*., 2002), and also can give useful information on the dissemination of populations of the pathogen (Zhang and Geider, 1997). According to these results, the strain isolated during this study, EPS101, could be connected to strains from the southern France and from the areas of Navarra, Euskadi and Aragon, and probably it was introduced from these areas.

Because few differences were observed among *E. amylovora* strains on the basis of cultural, nutritional and molecular traits and greater differences were observed in virulence in immature pear fruits, a more exhaustive study of the virulence was performed in order to select a strain or a group of strains to carry out the screening of potential biocontrol agents,

and also to optimize the pathosystem models based on blossoms and shoots.

Studies on the virulence are generally scarce because most studies on pathogens, especially *E. amylovora*, are oriented to understand the function of virulence factors and genes involved (Bellemann and Geider, 1992; Tharaud *et al*., 1994; Wei and Beer, 1995; Bogdanove *et al.*, 1998; Kim and Beer, 1995). Nevertheless, some studies have already been performed describing the virulence among *E. amylovora* strains (Norelli *et al*., 1984; Manulis *et al*., 1998) and the existence of slight host species-specific relationship (Momol and Zeller, 1993; Kim *et al.*, 1995).

Before the initiation of a virulence study, factors that can influence virulence must be taken into account like the pathogen concentration, the susceptibility of the plant material and host. The susceptibility of the host and plant material (micropropagated) can be standardized by means of the use of homogenous material from the same cultivar. In contrast, the proper pathogen concentration may depend on strain virulence and strongly affects disease development. Thus, studies are necessary to determine the effect of concentration on disease development (Johnson, 1994; Montesinos and Bonaterra, 1996; Bonaterra *et al*., 2003). In addition, the preliminary assay performed in immature pear fruits showed that virulence differences according to maximum disease incidence were not observed when fruits were inoculated at relatively high pathogen concentrations (10^6 and 10^7 cfu·mL^{-1}) which unable to find difference in highly virulent strains or highly susceptible cultivars. Therefore, a more detailed study of the effect of the pathogen concentration was also necessary in order to determine the suitable concentrations. The study was focused on the determination of the DE_{50} and *MID* from dose-disease relationships of 5 strains of *E. amylovora*. ED_{50} and *MID* depends on the time at which disease was assessed, reason why dose-response curves were fitted at different times, observing that models fitted reasonable well with the data collected after 5 days of pathogen inoculation. ED_{50} and *MID* were estimated by non-linear regression using two models, the probit and the hyperbolic saturation (Montesinos and Bonaterra, 1996) and results were similar to those obtained by linear regression. ED_{50} and *MID* values were similar among strains and only strain NCPPB595 presented significantly higher values indicating that this strain is less virulent. In contrast, strain UPN529 was the most virulent considering the *MID*, while the strain EPS101 was the most virulent considering the ED_{50}. Although, the classification of *E. amylovora* strains according to their ED_{50} and *MID* was similar, a greater variability among strains was observed in *MID* which indicates that working to low doses is easier to find differences. Therefore, a pathogen concentration near to the *MID* was selected to perform the virulence study in the more extensive collection of *E. amylovora* strains. Nevertheless, a higher dose was selected, around ED_{50}, in order to observe differences among the less virulent strains.

Data were analyzed deeply in a more accurate study using disease progress curves. These curves represent the integration of host, pathogen and environmental effects during the course of disease development (Campbell and Madden, 1990). Early models of disease progress curves, which depict changes of disease intensity with time, were introduced by Large (1952), Barrat (1945) and Van der Plank (1963). These and other models have been

reviewed by Zadoks and Schein (1979), Madden (1980), Kranz and Rotem (1988) and Kranz (1990). Model selection is important because the parameters estimated for the model are used in statistical analysis and comparison of disease progress curves (Campbell and Madden, 1990). The most used models are the exponential, monomolecular, logistic and Gompertz. Although, the Gompertz model usually is the most suitable to fit disease progress curves (Madden, 1986). All these models were tested in the present work confirming that the modified Gompertz model (with the addition of the parameter describing the onset of infection, t_0) provided the fit to experimental data.

Quantitative parameters estimated from disease progression curves using the modified Gompertz model were related to virulence such as the maximum disease proportion (I_{max}), apparent rate of disease progression (r_g) and onset of infection (t_0) (Van der Plank, 1963). However, each parameter provides specific information. Considering "infectivity" as the ability of the strains to initiate and produce infections, and "aggressiveness" as the rapidity in developing symptoms and disease progression, r_g and I_{max} allow the differentiation of *E. amylovora* strains according to their "aggressiveness", while t_0 allow the differentiation according to their "infectivity". Another parameter is *AUDPC* that is also appropriate for comparison because integrates the information provided by the other parameters (Madden, 1983; Berger, 1988; Campbell and Madden, 1990).

Significant differences were observed among strains for the four assessed parameters (I_{max}, r_g, t_0, *AUDPC*) at 10^5 cfu·mL^{-1} allowing to classify strains in three virulent groups (low, moderate and high). F values were lower for the I_{max} (F=35.12) than for t_0 (F=317.72) which indicates that I_{max} is less discriminant than the other parameters, and for this reason differences among strains are less evident. However, I_{max} is the easiest parameter to be calculated. Most of strains were classified at similar level of virulence for all the parameters, but some strains presented differences on virulence levels using one or another parameter. For example, strains USV4300 and USV4320 presented a high virulence according to r_g, while a moderate virulence on the basis of t_0. Therefore, these strains are very aggressive once they have initiated the infection, but present a low infectivity. In contrast, strains OMP-BO1185 and EAZ4 were highly infective according to t_0 but low aggressive according to r_g.

The most aggressive strain was USV4320 because present a high r_g at both doses, while the most infective strain was CFBP1430 exhibiting the lowest t_0 at both doses. In contrast, the strains less virulent were NCPPB311, UPN546, UPN609, UPN610 and EPS100 presenting the worst values for almost all the parameters. Peculiarly, most of strains with low virulence in immature pear fruits had been isolated from ornamental plants, which could be in concordance with the existence of host specificity described by other workers (Norelli *et al*., 1984; Beer *et al*., 1996).

Due to the fact that each virulence parameter give different information, the *CVI* was calculated in order to integrate the information provided by each one. The classification obtained was similar to that obtained according to *AUDPC*. This is obvious because *AUDPC* integrates the information of I_{max}, r_g and t_0. Therefore, the *AUDPC* may be sufficient if the

objective is simply to calculate the virulence of strains without taking in consideration aggressiveness or infectivity, and calculation of *AUDPC* does not require fitting data to mathematical models.

At the global level, the most virulent strains were UPN513, USV2773, USV4512, USV4576, USV4501, USV1043, UPN544 and EPS101, and the less virulent strains were NCPPB311, UPN546, UPN609, UPN610 and EPS100. Nevertheless, strains USV4501, USV1043, UPN544 and EPS101 presented high virulence at 10^5 cfu·mL^{-1} but lost virulence at low doses (10^3 cfu·mL^{-1}), while strains UPN513, USV2773, USV4512, USV4576 maintained a high virulence at low doses.

The second objective of this chapter was the optimization of model pathosystems to be applied to the selection of a biocontrol agent of fire blight, and in interaction studies. Two models systems were assessed, the blossom and the shoot assay. The completion of the blossom assay was based on the method described by Pusey (1997) but some modifications were introduced in order to adapt the method to our necessities. Two inoculation methods (drop deposition and rubbing with soaked paintbrush) and two pathogen concentrations (10^7 and 10^8 cfu·mL^{-1}) were evaluated. Concerning the inoculation methods, no differences in disease levels were observed between treatments, therefore the method based on drop deposition was selected because it permit a greater precision of volumes and concentrations of inoculum and is a method more approximate to the natural ways of infection. In relation to concentration of the pathogen, greater levels of disease incidence and severity were observed at 10^8 cfu·mL^{-1} (around 100%) than at 10^7 cf·mL^{-1} (around 80%). Therefore the low concentration was selected to perform the efficacy assay in order to favor the antagonistic activity due to the fact that flowers are a material too sensible and because the disease levels were reasonably high and this concentration is the standard used in all the bioassays performed in the present study. This was in accordance to other bioassays performed by other researchers (Rundle and Beer, 1987; Wilson *et al*., 1992; Pusey, 1997; Tharaud *et al*., 1997).

The achievement of the shoot assay was based on the method described by Tharaud *et al*. (1997). For the shoot blight assay, the pathogen was inoculated by the deposition of 10 µL of pathogen suspension on the wounds produced on the leaves. Two pathogen concentrations (10^6 and 10^7 cfu·mL^{-1}) were assessed in order to select the most suitable concentration for the development of efficacy assay. Strain EPS101 inoculated at 10^7 cfu·mL^{-1} developed high levels of disease incidence and severity without variability between repetitions. In contrast, when the pathogen was inoculated at 10^6 cfu·mL^{-1} an excessive variability of the results between repetitions were obtained. So the inoculation of the pathogen was performed at 10^7 cfu·mL^{-1}, where the results obtained were more consistent and without significant differences between incidence and severity, and because the disease levels were enough high to perform efficacy evaluations.

From this chapter, it was concluded that *E. amylovora* strains analyzed in the present study appeared very homogeneous according to cultural characteristics, PCR and ELISA reaction,

metabolic profiles based on API 20NE and Biolog GN systems, intensity of the infections in immature pear fruits and genetic diversity by RFLPs of *ams* gene and genomic DNA. In contrast, differences in the virulence of strains were found on the basis of parameters estimated from dose-disease relationships and disease progression curves. These parameters allowed the classification of strains according to their aggressiveness (ED_{50}, I_{max}, r_g), infectivity (*MID*, t_0), or general virulence (*CVI*, *AUDPC*). In the present work, immature pear fruit, blossom and shoot infection assays have been optimized using the deposition of 10 µL of a suspension adjusted at 10^7 cfu·mL^{-1} on wounds produced in the leaves for the shoot assay or on the hypanthium surface of the flowers for the blossom assay. Finally, strains used in the selection of potential biocontrol agents of fire blight were chosen mainly on the basis of their virulence. Two strains were selected, the strain CUCM273 because it is one of the most divergent *E. amylovora* strain due to it is not detectable by DASI-ELISA, does not present the typical white, domed, shiny, mucoid colonies (levan type) in sucrose nutrient agar due to it does not produce the amylovoran; and the strain EPS101 because it presents the common characteristics of most of *E. amylovora*.

CHAPTER 3

Selection of potential biocontrol agents of *Erwinia amylovora*

INTRODUCTION

Fire blight can be partially controlled through the use of appropriate cultural measures and repeated applications of antibiotics, such as streptomycin and kasugamycin, and by copper derivates or other compounds. Although, many chemical agents have low activity or cause phytotoxicity, and streptomycin, the most effective, is not approved for use in many countries (Wilson *et al*., 1990). In addition, the use of antibiotics in the countries where they are permitted allowed the selection of resistant strains that are becoming more widespread, limiting the disease control. As a result, in 1993 biological control using epiphytic bacteria was already considered a promising alternative to chemical control (Wilson and Lindow, 1993).

It is relatively easy to isolate potential biocontrol candidates, but it is difficult to obtain microbial antagonists that are sufficiently effective in the control of *E. amylovora* with consistent results under field conditions. The first step in the development of a biological control program is the constitution of a large isolates collection and the later selection of isolates with ability to inhibit the pathogen or the disease it causes (Andrews, 1992). An important and necessary step in the biocontrol agent selection process is the characterization of the isolates. This characterization is necessary to select those with interesting treats and to discard others with deleterious or non interesting properties such as ice nucleation activity or being pathogenic. Besides, characterization can be useful to determine the implication of some traits in the biocontrol activity and improve selection procedures.

The selection process is possibly the most difficult step in a biological control program development. Besides, it is important to evaluate the efficacy, growth rate, and colonization at an early stage of the screening process. In this sense, field trials are the most reliable tests but are expensive, so only can be employed for a reduced number of pre-selected antagonists. In order to test a large number of potential antagonists and to reduce the time

and work effort involved in the screening process, rapid initial tests of antagonism are necessary.

The selection process of biological control agents of fire blight is limited by the lack of information of the possible mechanisms involved. Antagonist efficacy is generally mediated by one or more mechanisms like the production of antibiotics, competition for nutrients, blockage of entrance sites, acidification of the environment, or host activation of defense mechanisms (Wright and Beer, 1996). Therefore, it is necessary to evaluate the biological control efficacy of hundred or thousands strains using *in vitro* or *in vivo* assays.

Strategies for the isolation and testing of biocontrol agents of *E. amylovora* have been discussed and improved screening procedures have been developed. First studies screened potentially antagonistic strains of *E. herbicola* on the basis of their ability to produce antimicrobials *in vitro* (Beer *et al*., 1984b; Lindow, 1988). However, the screening assay based on growth inhibition of *E. amylovora* on Petri plates is rapid but it only allows to select antibiotic producing strains and many antagonists are discarded. Besides, there is no correlation between inhibition on Petri plates and the ability to inhibit the pathogen in the field (Beer *et al*., 1984b).

The *ex vivo* assays were later developed as an advance on screening methods. The first *ex vivo* assay developed was the immature pear fruit assay (Beer and Rundle, 1983). This assay was first developed to test pathogenicity of suspected strains of *E. amylovora* (Billing *et al*., 1960), but it was later modified to screen for potential biocontrol agents of fire blight. This assay was considered more appropriate than culture media-based tests because is more representative of the natural interaction. Immature pear fruit test permits the selection of antagonists of *E. amylovora* regardless of the mechanism of control, prevent premature discard of potential antagonists and it is enough restrictive to reduce selection of false-positive isolates. Although, this method presents some problems of heterogeneity of results and lack of consistent correlation with disease control in orchards (Vanneste, 1996; Pusey, 1997; Mercier and Lindow, 2001).

Discrepancies between such inhibition tests and performance in disease control by antagonists of *E. amylovora* have been found (Wilson *et al*., 1990). Some researchers have found that laboratory-based assays with culture media or fruits did not always correlate with biological control effectiveness on blossoms (Wilson *et al*., 1992; Mercier and Lindow, 1996). Nonetheless, these tests are proposed as the pre-screening step to discard most of isolates and only select the most promising candidates. Such pre-screened potential biocontrol agents must then be subjected to a more exhaustive empirical selection procedure involving tests on blossoms and plants under controlled environment or field conditions to verify their efficacy. The second selection level takes into account other traits which are also likely to be involved, such as the ability to be competitive in complex microbial communities, or the ability to colonize plant tissues. These traits will be important in a successful biocontrol agent, but are difficult to quantify on plate-agar assays or in simple immature pear fruit assays.

It has been developed a crab apple blossom assay to screen potential biocontrol agents under controlled environment conditions (Pusey, 1997). This assay is based on the assumption that disease incidence is directly related to the population level of the pathogen on stigmas. This method allows the identification of new biological control agents and perhaps new mechanisms, and it is an alternative to immature pear fruits assay.

The young shoot assay was developed as an advance on *ex vivo* assays. It is known that *E. amylovora* is not a very good epiphyte and their population levels on leaves and flowers decline rapidly within a few hours or days under natural conditions. However, it is possible to find transitory populations of *E. amylovora* on leaf surfaces after blossom infections occurred in the orchard, and large numbers of *E. amylovora* were found on the leaves shortly after the apparition of fire blight symptoms (Vanneste and Eden-Green, 2000). Biological control of these epiphytic populations of *E. amylovora* can suppose the reduction of blossom and shoot blight. Accordingly, the test of inhibition of infections of young shoots includes the interaction between the full plant, the pathogen and the biocontrol agent, and allows the determination of the ability of the antagonists to colonize the plant surfaces and antagonize with the *E. amylovora*.

Thus, there are several screening methods developed to identify potential biocontrol agents of fire blight and also there are several biocontrol agents of fire blight described that have been obtained by means of different screening procedures. However, there are no reports on existing relationships between control efficacy and relevant characteristics in wide collections of fire blight biocontrol agents

OBJECTIVES

The present study was conducted to determine traits displayed by *E. amylovora* antagonists that can be related to their efficacy in the inhibition of infections and to develop a methodology of selection based on this acquired knowledge.

Specific objectives were to:

- Isolate epiphytic bacteria from several host plants, and screen for infection inhibition by *E. amylovora* in immature pear fruits.
- Identify at the species level the most effective selected antagonists by means of biochemical and microbiological tests.
- Characterize the selected antagonists by means of in vitro antagonism tests, production of antimetabolite compounds and absence of deleterious or plant pathogenic characteristics.

- Determine the existence of specific traits or patterns displayed by the antagonists associated to inhibition of *E. amylovora* infections in immature pear fruits.
- Select a putative biological control agent based on efficacy and consistency of inhibition of infections of *E. amylovora* in immature fruits, flowers and young shoots.

MATERIALS AND METHODS

1. Build-up of a collection of bacterial isolates

The Centre for Innovation and Development in Plant Health (CIDSAV) already had an isolates collection though, in this work, the collection was extended with new isolates obtained from a specific guided isolation with the purpose to improve the amount of fire blight antagonists. New isolates were collected from the same environment where the fire blight develops, mainly from aerial parts of Rosaceous plants, following two isolation procedures, a species oriented procedure and an *ex vivo* selective enrichment procedure.

The species oriented procedure was based on colony growth in differential media which permit to distinguish roughly species of fluorescent *Pseudomonas* (King's B medium) and *Erwinia* (MS medium) (*Annex 2*). Putative bacterial antagonists were isolated from 39 samples of leaves and roots of several Rosaceous plants from rootstocks in a commercial nursery (Agromillora Catalana S.A., Barcelona, Spain) (*Annex 4*). Roots were gentle washed with sterile distilled water to remove soil debris. Epiphytic bacteria were extracted from plant material that was homogenized in buffered peptone water (*Annex 1*) by a stomaker homogenizer (Masticator, IUL Instruments, UK) (*Figure 3.1*). Extracts were 10-fold serially diluted and spread in 0.1 mL aliquots on plates of KB and LB (*Annex 2*) amended with 50 $\mu g \cdot mL^{-1}$ cycloheximide to avoid fungal growth. Plates were incubated for 48 h at 25 ºC. Potential isolates of *P. fluorescens* were identified in KB agar as fluorescent colonies under ultraviolet light (366 nm), and in the case of *E. herbicola* were identified as typical orange colonies in LB agar (*Figure 3.2*). Single colonies were streaked on LB agar to get pure cultures. Although, they were not formally identified at this stage but the isolates were referred as *E. herbicola* and *P. fluorescens*. For long-term preservation, bacteria were stored in 20% glycerol at –80 ºC. This group was composed of 169 isolates that were obtained during this study (*Annex 5*).

The *ex vivo* selective enrichment procedure was based on the selection of isolates from mixed microbiota enrichments of extracts that inhibited the development of *E. amylovora* infections when were applied on wounded immature pear fruits inoculated with 10 µL of *E. amylovora* suspension adjusted to 10^7 $cfu \cdot mL^{-1}$. The isolates were obtained from 64 enrichments from plant samples which inhibited infections as previously described. Representative colonies were collected from agar cultures, including fungi, yeasts and

bacteria. A total of 276 isolates were obtained using this procedure in a previous study (*Annex 6*) (Vázquez, 1997).

Figure 3. 1. Homogenization of plant material by a homogenizer of shovels.

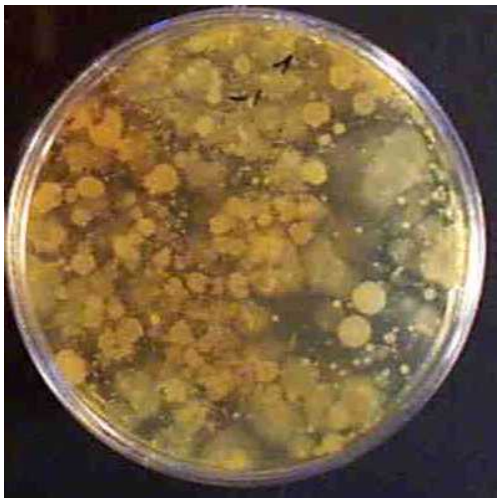

Figure 3. 2. Direct extract cultured in King's B medium agar.

Also 79 antagonistic strains of *E. amylovora* in immature pear fruits that proceeded from the collection of the CIDSAV were included in the collection (*Annex 7*). These strains were identified as *E. herbicola* and *P. fluorescens* using the API 20E system (Biomerieux, Marcy l'Etoile, France), and were included because they present a high efficacy in the inhibition of *E. amylovora* infections in immature pear fruits (Bonaterra, 1997). Besides, 11 reference strains kindly provided by different researchers were added: *P. fluorescens* BL915 (Steve Hill, Novartis Crop Protection, Inc. Research Triangle Park, North Carolina, USA), *P. fluorescens* CHA0 (Gènieve Défago, Institute of Plant Sciences/Phytopathology, Swiss Federal Institute of Technology, Zurich, Switzerland), *Pseudomonas* sp. JBR1-70 and JMP12-84 (Jos J. Raaijmakers, Laboratory for Phytopathology, Wageningen Agricultural University, The Netherlands), *P. fluorescens* Ps15 and Ps31 (Mette Neiendam Nielsen, Departement of Ecology and Molecular Biology. Section of Genetics and Microbiology, Royal Veterinary and Agricultural University, Denmark), *Pseudomonas* sp. WB1(Walid Fakhouri, Universität Hohenheim, Institut für Phytomedizin, Stutgart, Germany), *P. fluorescens* SBW25 (Mark J. Bailey (Molecular Microbial Ecology, Institute of Virology and Environmental Microbiology, Oxford, England), *P. fluorescens* Q2-87, Q4-87 and 2-79 (Linda Thomashow, USA Department of Agricultural Research Service, Root Disease and Biological Control Research Unit, Washington State University, USA). Also two reference strains from the "Colección Española de Cutivos Tipo" (CECT) (CECT378, CECT844), and four from the "Collection Francaise des Bactéries Phytopathogénes" (CFBP) (CFBP2128, CFBP2131, CFBP2126, CFBP2129) were included. Therefore, a very wide collection of 535 strains was finally build-up for the present study (*Table 3.1*).

Table 3.1. Number of isolates composing the collection according to species

Source	Number of isolates	*P. fluorescens*	*E. herbicola*	Others (not determined)
Species oriented procedure	169	92*	45*	32*
Ex vivo selective enrichment procedure	276	9	14	234
Antagonistic strains from CIDSAV collection	79	40	39	-
Biocontrol and reference strains	11	11	-	-
Total	535	152	98	266

*, results obtained during the present study.

2. Screening of *E. amylovora* antagonists

The 535 isolates predominantly *E. herbicola* and *P. fluorescens*, obtained from roots, flowers, fruits and leaves of rosaceous plants, were screened using the immature pear fruit assay.

Maintenance and growth of putative antagonists

Bacterial and yeast isolates were cultured overnight at 25 ºC in 20 mL of LB broth. Bacterial suspensions were centrifuged at 5.000 g during 20 min and the pellet was suspended in distilled water. Final suspensions of 200 mL were adjusted turbidimetrically (Shimadzu Corporation UV-160A, Kyoto, Japan) to 10^8 cfu·mL^{-1} for bacteria and 10^6 cfu·mL^{-1} for yeast and fungi. Conidial suspensions of fungal isolates were obtained from pure cultures grown in potato dextrose agar (PDA) (*Annex 2*) during 7 to 10 days. Conidia were collected by scraping the culture surface with a wet cotton swab and suspending the material in distilled water containing Tween20 at 5‰. Fungal suspensions were adjusted with an hemocytometer at 10^6 conidia·mL^{-1}. Bacterial suspensions were adjusted turbidimetrically to the appropriate cell concentration. The suspensions were stored at 4 ºC until use, but this time never overcame two hours.

Pathogen strain

E. amylovora strain CUCM273 was used as the pathogen. It was isolated from Greening apple in North America (Prof. S. Beer, Cornell University Collection of Microorganisms, Ithaca, USA, 1971). This strain was characterized in the present study by means of the API 20E system, growth in different culture media, PCR with specific primers, ELISA-DASI enrichment, MRFLP's through PFGE and virulence in immature pear fruits (*Section 1.2.3* from *Chapter 2*). For inoculation, the pathogen was cultured overnight at 25 ºC in LB plates, and cultures were scraped from the agar surface and suspended in distilled water. The suspension was adjusted turbidimetrically to 10^7 cfu·mL^{-1}.

Plant material

Detached immature pear fruits of Passe Crassane cultivar were obtained from a commercial orchard near Girona. Fruits aged 6 weeks were collected in June and kept in the dark from 0 to 4 ºC with moderate aeration under high relative humidity. Fruits were surface disinfested by immersion for 10 min in a solution of sodium hypochlorite (1%) and washed two times in sterile distilled water and let stand to remove moist. Wounds were done, four in each fruit, with a tip (approximately 5 mm depth and 3 mm wide) before the treatment with putative antagonists.

Immature pear fruit assay

The method described by Beer and Rundle (1983) was modified so that whole pears with four symmetrical wounds were used instead of halves. Pears were treated with putative antagonists by immersion in 200 mL of the corresponding isolate suspension adjusted to the suitable concentration during 15 min (*Figure 3.3*). The fruits were placed in polystyrene tray packs covered by plastic bags (*Figure 3.4*). After 24 h of incubation at 21 ºC, each wound was inoculated by depositing 10 µL of a pathogen suspension of 10^7 cfu·mL^{-1}. Pathogen inoculations and disease assessment were always done under biosafety conditions within a class II biological safety cabinet (Nuaire Class II UN-426-400E, Nuaire Inc., USA) (*Figure 3.5*).

Figure 3.3. Treatment of immature pear fruits by immersion in suspensions of the isolates.

Figure 3.4. Immature pear fruits with four wounds placed in tray packs.

Each treatment consisted of 3 repetitions of 3 fruits per repetition. Treatments were completely randomized. Two non-treated controls treated with water were included, one inoculated with the pathogen and another without the pathogen inoculation. Pears were incubated in plastic boxes, closed hermetically in security plastic bags and incubated in an expression chamber (Growth Cabinet MLR 350, Sanyo, Japan) at 100% of relative humidity with 16 h of fluorescent light and 21 ºC (*Figure 3.6*). Disease incidence was assessed for each repetition of 3 fruits and 4 wounds per fruit. Infection in a wound was considered when drops of bacterial exudates and/or necrosis were detected in and around after 9 days of incubation. Inhibition efficacy was calculated for each replicate and expressed as follow:

$$Efficacy\ (E) = \left(\frac{I_c - I_t}{T} \right) \cdot 100$$

where E is the efficacy of incidence reduction for each treatment, I_c the number of infected wounds in the non-treated pathogen inoculated control, I_t the number of infected wounds in the treatment, and T is the number of total wounds (12 wounds) for the immature fruits corresponding to each repetition.

Figure 3.5. Inoculation of immature pear fruits with *E. amylovora* in a biosafety cabinet using an automatic inoculator.

Figure 3.6. Incubation of immature pear fruits after antagonist treatment and pathogen inoculation.

Data of efficacy were analyzed by means of an analysis of variance (ANOVA) and Tukey's mean separation test using PC-Statistical Analysis System (SAS) version 8.2 (SAS Institute Inc., Cary, NC, USA).

3. Identification and characterization of potential biocontrol agents

The 61 strains that showed the highest effectiveness in the inhibition of infections in immature pear fruits were selected from the 533 initial isolates (*Annex 8*). Selected strains were identified using API strips, and characterized for *in vitro* antagonism on agar media against different plant pathogens, production of antimetabolite compounds (antibiotics, siderophores, chitinases, indolacetic acid), inhibition of infections in immature pear fruits, hypersensitivity reaction and ice nucleation activity.

3.1. Identification of the best isolates

P. fluorescens were preliminary identified on KB agar as fluorescent colonies under ultraviolet light (366 nm). *E. herbicola* were preliminarily identified by typical orange colonies in LB agar. *Serratia marcescens* isolates were identified by typical red colonies in LB agar. The final identification was based on the results obtained by Gram stain, cell morphology, motility, and API system.

Gram reaction, morphology and motility

An alternative to gram stain was used based on the method of Ryu Potasse test consisting of the solubility of the gram negative colonies in 3% KOH (Suslow *et al.,* 1982). Cell morphology was observed using phase contrast microscopy (BH2, Olympus, Tokio, Japan), and the motility was determined using the test of the hanging drop.

API 20E and 20NE systems

Selected isolates were identified using the API system (Biomerieux, France). Strips were used following the indications of the supplier. API 20E galleries were used to identify the isolates that presumably corresponded to *E. herbicola* and *S. marcescens* species, because these strips are recommended for the Enterobacteriae family. The strips contained 20 biochemical standardized tests including β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophane deaminase, indole production, acetoin production, gelatinase, glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, arabinose fermentation or oxidation. The isolates that presumably corresponded to *P. fluorescens* were identified using API 20NE system containing 21 biochemical standardized test including the reduction of nitrates to nitrites, reduction of nitrates to nitrogen, indole production, glucose acidification, arginine dihydrolase, urease, β-glucosidases hydrolysis, protease hydrolysis, β-galactosidase, glucose, arabinose, mannose, mannitol, M-acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate, phenyl-acetate assimilation, cytochrome oxidase. Species identification by means of the resulting code was made using the corresponding data base of the API system.

Biochemical tests

Independent biochemical tests of the API auxanographic system were made. Oxidative-fermentative metabolism and oxidase test were added. Tests were described in *Table 3.2.*

Table 3.2. Description of oxidative-fermentative metabolism test and oxidase test according to medium used, revelation reagent and results

Test	Medium	Reagent	Detection	Reference
Oxidative-fermentative metabolism	2g Peptone, 5g NaCl, 0.3 g K_2HPO_4, 10 g glucose, 60 mg bromotimol blue, and 5g agar in 1 litter of water	---------	yellow	Hugh and Leifson, (1953)
Oxidase	----------	1% of tetramethyl- p-phenilendiamine	blue	Kovaks, (1956)

3.2. Characterization of the antagonistic capacity of the strains

Selected strains were characterized in relation to the *in vitro* antagonism and production of antimicrobial compounds. The antagonism against *Xanthomonas campestris, E. amylovora* and *Pseudomonas syringae* was assessed in different agar media. The production of several compounds (antibiotics, siderophores, chitinases, indolacetic acid) and the pathogenic capacity through the hypersensitivity assay, and ice nucleation activity were also determined.

3.2.1. *In vitro* antagonism spectrum

Antagonism on agar media was assessed against *Xanthomonas arboricola* pv. *juglandis* 1317.2 (Instituto Valenciano de Investigaciones Agrarias), *E. amylovora* PMV6076 (Collection Française des Bactéries Phytopathogénes), and *Pseudomonas syringae* pv. *syringae* EPS94 (CIDSAV) and to the fungus *Penicillium expansum* EPSF (CIDSAV). All bacterial strains of pathogens were stored at -80 ºC in LB broth containing 20% glycerol. For use, cultures were grown overnight at 25 ºC in LB agar. Bacterial cells were removed from the agar surface and suspended in water. Suspensions were adjusted turbidimetrically to 10^8 cfu·mL^{-1}. Conidia of fungal target strains were obtained from pure cultures grown in PDA during 7 days, by scraping the culture surface with a wet cotton swab and suspending the material in distilled water containing Tween20 at 0.5‰. Concentration of conidia was adjusted with an hemacytometer at 10^5 conidia·mL^{-1}.

Antagonism on agar media against bacterial indicators was performed with agar overlays of glucose-asparagine (GA) (*Annex 2*), GA amended with iron (50 μM $FeCl_3$) and LB. GA was amended with iron to prevent siderophore production in bacteria that showed antagonism on GA agar. Antagonism against fungus was performed on LB, GA and PDA (*Annex 2*). Overlays were prepared by mixing 4.5 mL of melted agar and 0.5 mL of bacteria or fungus suspension of 10^8 cfu·mL^{-1} and 10^5 conidia·mL^{-1}, respectively. Antagonistic colonies were transferred to the surface of the overlay agar plates with toothpicks, and plates were incubated at 25 ºC. Three repetitions for each strain were performed. Antagonism was detected as the presence of a halus of inhibition of the indicator microorganism around the colony. The diameter of the inhibition zones was assessed after 24 and 48 hours (Montesinos *et al.,* 1996).

3.2.2. Production of antimicrobial compounds and other metabolites

The selected isolates were studied for production of known antibiotics, siderophores, chitinases, indolacetic acid, and cyanide by means of microbiological and molecular techniques.

Antibiotic production

The capacity of strains to produce several antibiotics like phenazine-1-carboxylic acid (PCA), 2,4-diacetylphlorogucinol (Phl), and pyrrolnitrin (Prn) was determined. An indirect qualitative method based on the detection of biosynthetic genes by PCR was used for each antibiotic. The oligonucleotide primers used for Phl detection were targeted to the *phlD* gene according to Raaijmaikers et al. (1997), one of the six biosynthetic genes described for *P. fluorescens* Q2-87 (Genbank accession n. U41818). In case of PCA, primers were developed from two genes described inside the PCA biosynthesis cluster of *P. fluorescens* 2-79 (*phzC* and *phzD*) (GenBank accession n. L48616) (Raaijmakers *et al.,* 1997). Prn primers were developed for the *prnD* gene that encode for the aminopyrrolnitrin oxidase (GenBank accession n. U74493) (Badosa, 2001).

Standard PCR were performed with the Phl, PCA and Prn gene primers shown in *Table 3.3.* PCR reaction was carried out in a volume of 25 µL, containing 5 µL of heat-lysed cell suspension, 2.5 µL of dNTPs 2 mM (Amersham Pharmacia Biotech, Sweden), 10 pmol of each primer, 1.5 µL Cl_2Mg 25 mM, 2.5 µL PCR buffer 10X, 11 µL of sterile demineralised water and 0.5 µL of Taq DNA polimerase 5 U μL^{-1} (Invitrogen Corporation,USA).

Amplifications were performed with a GeneAMP® PCR system 9700. The PCR program consisted of an initial denaturation step at 94 ºC for 2 min followed by 25 cycles of 94 ºC for 30 s, 67 ºC for 20 s, 72 ºC for 30 s, and a final elongation at 72 ºC for 10 min. Samples of the PCR products were separated by horizontal electrophoresis on a 1.2% agarose gel in 1X TAE buffer at 70 V for 45 min.

Table 3.3. Primers used for PCR analysis of known antibiotics

Primer	**Sequence 5'→ 3'**	**G+C** %	**Temperature** (ºC)	**Position**[a] (pb)	**Reference**
Phl2a	GAGGACGTCGAAGACCACCA	60	73	1915	Raaijmakers *et al.,* 1997
Phl2b	ACCGCAGCATCGTGTATGAG	55	72	2660	Raaijmakers *et al.,* 1997
PCA2a	TTGCCAAGCCTCGCTCCAAC	60	79	3191	Raaijmakers *et al.,* 1997
PCA3b	CCGCGTTGTTCCTCGTTCAT	55	76	4341	Raaijmakers *et al.,* 1997
Prna	TCAAGGACAAGCCGACCGAGT	57	68	5007	Badosa, 2001
Prnb	GCAGCCCGAACAGCACGAAGT	61	69	5837	Badosa, 2001

[a] position of the 5' end of the primer in the database sequence. Size of fragments amplified by primers: Phl, 745 pb; PCA, 1150 pb; and Prn, 830 pb.

The gel was stained with ethydium bromide for 30 min, and the PCR products were viewed with a UV transilluminator. The expected lengths of the fragments obtained by PCR were of 1150 for Phl, 745 for PCA and 1090 pb for Prn.

Siderophore production

Siderophore production was determined *in vitro* by means of the Chrome Azurol S (CAS) agar plate method as described by Schwyn and Neilands (1987). CAS agar media included a blue indicator that contains Iron (III), (CAS)-Iron (III)-bromide of hexadecyltrimethyl-ammonium (HDTMA). Siderophore captures Iron (III) and the indicator turns orange. Isolates were grown previously in KB media in order to stimulate the siderophore production and were inoculated in CAS agar plates by transferring the colonies to the surface of the agar plates with toothpicks. Plates were incubated at 22 ºC for 2 days. Isolates exhibiting an orange halo around the colony were considered positive for production of siderophore. Strain BL915 of *P. fluorescens* was included as a positive control. Three repetitions for each strain were performed.

Chitinase production

Two methods were used to test the qualitative production of chitinases. A microbiological method was based on growth of isolates in BBL medium amended with colloidal chitin (*Annex 2*) (Frändberg and Schnürer, 1998) The isolates were inoculated on chitin agar by transferring colonies to the surface of the agar plates with toothpicks and were incubated for 7 days. Isolates exhibiting a transparent halo around the colony were considered positive for production of chitinase. The strain EPS325e of *Serratia marcescens* was included as a positive control. Three repetitions for each strain were realized.

A second method was based on indirect detection by PCR. This method was targeted to genes that encode for chitinase. Nevertheless there are not sequences corresponding to chitinase genes of *P. fluorescens* or *P. agglomerans* deposited in the Genbank. For that reason, primers used were developed for the *chiA* gene of *S. marcescens* (GenBanK accession n. LO1455) (Chernin *et al.,* 1997). Sequences of the primers were 5'-TATCCTCTCGGAATAA-3' and 5'-GAATTCACTCAAACAATCT-3', and the expected length of the fragment obtained by PCR was of 2165 pb. PCR amplification was carried out as for in the production of antimicrobial compounds, only changing the PCR program that consisted of an initial denaturalization at 94 ºC for 2 min followed by 25 cycles of 94 ºC for 30 s, 54 ºC for 20 s, 72 ºC for 30 s, and a final elongation at 72 ºC for 10 min. Samples of the PCR products were separated on a 0.8% agarose gel in 1X TAE buffer at 70 V for 45 min. Products were viewed using the same procedure described previously.

Indolacetic acid production

An *in vitro* method was used for screen indolacetic acid (IAA) and analogue products (Bric *et al.,* 1991). Isolates were grown on LB amended with 5 mM 1-tryptophan that was overlaid with an 82-mm-diameter nitrocellulose membrane disk (Amersham Pharmacia Biotech, Spain). Agar plates were inoculated with bacterial cultures and incubated at 28 ºC for 3 days. The membranes were overlaid with a Whatman n.2 filter paper saturated with

Salkowski reagent (*Annex 2*). Microorganisms producing IAA or analog compounds exhibited a characteristic pink to red color after 0.5 to 3 h in the filter surface. Strain CHA0 of *P. fluorescens* was included as a positive control. Three repetitions for each strain were done.

Cyanide production

Hydrogen cyanide production was assessed by the picrate method (Sneath, 1966). The method is based on the growth of microorganisms on a 4 mL of LB medium where an impregnated strip of sterile paper with an alkaline picric acid solution (*Annex 2*) was introduced once the microorganisms were inoculated. Tubes were incubated during 3 days at 22-25 ºC. Positive results were obtained when the paper strip turned of yellow to orange-brown color. Strain CHA0 of *P. fluorescens* was included as a positive control. Three repetitions for each strain were performed.

3.2.3. Antagonism against *E. amylovora* in immature pear fruits

The most active strains were retested for their antagonism ability against *E. amylovora* in immature pear fruits. Instead of strain CUCM273, strain EPS101 of *E. amylovora* was used to perform this test. This strain was isolated during the present work from a symptomatic sample of fire blight provided by the Crop Protection Service of the Generalitat de Catalunya. The sample consisted of a Conference pear branch obtained from a fire blight infected orchard in Lleida. *E. amylovora* EPS101 has been characterized at different levels during this the present work (*section 1.2.3* from *Chapter 2*). The procedure employed was as described in *section 2* from this chapter. ANOVA was performed to test the effect of the biocontrol agents in the efficacy of reduction of infections in immature pear fruits. Means were separated using the Tukey's test with a significance of 0.05 to group the strains in function of their virulence.

3.2.4. Multivariate analysis

An aggregation analysis was performed for the 61 strains using data of the API system and biochemical tests, *in vitro* antagonism and production of antimicrobial compounds, and infection inhibition of *E. amylovora*. The similarity matrix between strains was calculated using the Euclidean distance. Cluster analysis was performed by applying the Agglomerative Hierarchical group method through CLUSTER ANALYSIS procedure of S-PLUS. All the characteristics of the isolates were also compared by multivariate analysis. Correspondence analysis was used to describe the relationships between the characteristics and the efficacy of inhibition of infections by *E. amylovora* in immature pear fruits using the procedure CORRESP of SAS (Version 8.2, SAS Institute Inc., NC, USA). The following variables with the corresponding categories were used: bacterial species (*E. herbicola*, *P. fluorescens or S. marcescens*); plant host species (rosaceous or no rosaceous plants); plant organ source (rhizosphere or phyllosphere); antagonism against *E. amylovora* and *P. syringae* on LB, GA

and GA-fe (presence or absence); antagonism against *X. campestris* on LB (presence or absence); antagonism against *P. expansum* on LB, GA and PDA (presence or absence); IAA, HCN, chitinases, siderophores, and antimicrobial compounds production (presence or absence), and efficacy in inhibition of *E. amylovora* infections in immature pear fruits (low, moderate or high).

3.3. Deleterious effect tests

Hypersensitive tobacco response and ice nucleation activity were evaluated for the 61 strains to prevent selection of antagonists harboring deleterious characteristics.

3.3.1. Tobacco hypersensitivity response

Hypersensitivity response (HR) shows the potential of bacterial isolates to be pathogenic. This test is based on the capacity of non host plants to respond against the phytopathogens by means of an incompatible reaction. HR has been defined as localized cell death in an area of pathogen invasion, with plant tissue necrosis. The test is carried out with tobacco plants (*Figure 3.7*), but also can be performed on geranium plants (*Figure 3.8*). Suspensions of bacteria were adjusted turbidimetrically to 10^8 cfu·mL^{-1} from pure cultures grown during 24 to 48 h on KB medium. Inoculations were done on young tobacco plants of Xanthi cultivar. Suspensions were infiltrated into the intercellular space of the reverse of a leaf using a hypodermic needle. The plants were incubated at 20 ºC, 70% of relative humidity, and a photoperiod of 16 h of light. Symptoms were evaluated 24-48 h after the infiltrations. Complete collapse of the tissue after 24 h is recorded as positive. *P syringae* pv. *syringae* EPS94 (Moragrega, 1997) and *P. fluorescens* EPS200 were included as positive and negative controls, respectively. Three repetitions for each strain were done.

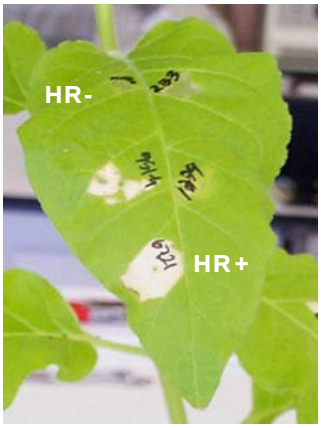

Figure 3.7. HR expressed on Tobacco.

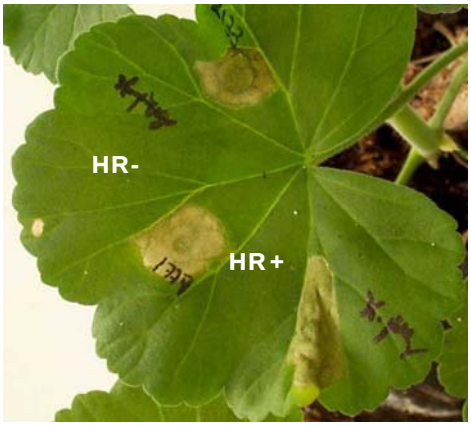

Figure 3.8. HR expressed on Geranium.

3.3.2. Ice nucleation activity

Ice nucleation activity (INA) is the process by which some bacteria can act as ice nucleus (INA^+), promoting the ice crystal formation at temperatures over the common temperature where the phenomenon occurs. INA was determined using a simple test, where bacterial suspensions in pure water at 10^8 cfu·mL^{-1}, were stored at 0 to 4 ºC from 1 to 2 h to stimulate the INA and then were submitted to a temperature gradient from 0 ºC to –10 ºC along 20 min inside a cryogenic alcohol bath (CB8-30E, Heto, Allerod, Dinamarca). Three repetitions for each strain were done. Negative controls with pure water and positive controls with *P. syringae* pv. *syringae* EPS94 were included. Strains that freeze suspensions during incubation were considered positive.

4. Selection of a biological control agent

The selection of a biological control agent was based on the criteria of efficiency and consistency in the suppression of blossom blight and shoot blight under controlled conditions.

4.1. Control of blossom blight

Eight antagonists, selected on the basis of their efficacy and consistency in the inhibition of *E. amylovora* infections in immature pear fruits of Passe Crassane cultivar (*section 3.2.3*) were used for the experiments of control of blossom blight under controlled environment conditions using the assay described by Pusey (1997).

Bacterial strains

Four strains corresponded to *P. fluorescens* (EPS62e, EPS538, EPS684 and EPS734), and four to *E. herbicola* (EPS438, EPS460, EPS156e and EPS283e). All strains were isolated from Rosaceous plants, but they were obtained from different plant host species, and plant organs (leaf, fruit, bud, or root) (*Annex 8*). Also it was verified that did not present any pathogenicity by means of hypersensitive response test in tobacco plants, and they were not ice nucleation active (*Table 3.12, 3.13* and *3.14*). Strain EPS101 of *E. amylovora* was used as the pathogen in this assay. All bacterial strains were stored in LB broth containing 20% glycerol at –80 ºC. Cell suspensions were prepared following the procedure described previously (*section 3.2.2*).

Plant material

Individual detached pear flowers of cultivar Doyenne du Comice obtained as previously described in the *section 4.1* from *Chapter 2* were used (*Figure 3.9*).

Antagonist treatment and pathogen inoculation

Two trials were done. In the first trial, eight strains were tested, four corresponding to *P. fluorescens* (EPS62e, EPS538, EPS684 and EPS734) and four corresponding to *E. herbicola* (EPS156e, EPS283e, EPS438 and EPS460). In the second trial only the highest efficient strains were used, four corresponded to *P. fluorescens* (EPS62e, EPS684 and EPS734), and one to *E. herbicola* (EPS283e). Individual detached flowers were submerged through the peduncle in a 1.5 mL Eppendorf containing a sucrose dilution (10%) and arranged in tube racks, and were sprayed with antagonist suspensions at 10^8 cfu·mL^{-1} (*Figure 3.10*). Flowers were covered with plastic bags and after 24 h of incubation at 21 ºC, high relative humidity and 16 h of fluorescent light, were inoculated by deposition on the hypanthium of 10 μL of a pathogen suspension at 10^7 cfu·mL^{-1}. Inoculation was done in a Class II biological safety cabinet. Flowers were covered with plastic bags, and incubated at 21 ºC, high relative humidity and 16 h of fluorescent light.

Figure 3.9. Detached pear branches with flowers buds forced to bloom.

Figure 3.10. Treatment of detached pear flowers disposed in tube racks.

The experimental design consisted of three repetitions of 8 flowers per repetition. Non-treated controls inoculated with water or with the pathogen were included. Disease incidence and severity per repetition were evaluated after 8 days of pathogen inoculation and calculated using the equations described in the *section 4.1* from *Chapter 2*.

Statistical analysis

ANOVA was performed to test the effect of putative biocontrol agents in the reduction of disease incidence and severity of blossom blight. The means were separated according to the Tukey's test at $P \leq 0.05$.

4.2. Control of shoot blight by strain EPS62e

The efficacy of EPS62e was assessed in the suppression of shoot blight under controlled environment conditions. This assay is useful to verify the potentiality of the selected strain to be effective in conditions closer to the orchard.

Bacterial strains

The biocontrol strain EPS62e that was isolated from an asymptomatic pear fruit, was assessed in the control of shoot blight caused by *E. amylovora* EPS101. Bacterial suspensions were prepared following the procedure previously described (*section 3.2.2*).

Plant material

Two types of plant material were used in the trials. In the first trial, young shoots from vegetative buds contained in Doyenne du Comice detached branches obtained as previously described in the *section 4.1* from *Chapter 2* were used (*Figure 3.11-A*).

In the second and third trials, self-rooted pear plants of cultivar Conference (CAV clone) obtained by micropropagation (Agromillora Catalana, S.A., Barcelona, Spain) were used. Plants were 2 to 3 years old, grown in 20-cm-diameter plastic pots. Plants were left outside the greenhouse during winter for chilling. During early spring, plants were pruned to leave 3 or 4 shoots and were forced to bud in the greenhouse. Plants were fertilized once a week with a 200 ppm N-P-K solution (20-10-20) and were used when the shoots were about 3 or 4 cm length and had 5 or 6 young leaves, which were the most susceptible to *E. amylovora* infection (*Figure 3.11-B*). Standard insecticide and miticide sprays were applied.

Biocontrol treatment and pathogen inoculation

Three trials were performed to determine the efficacy of EPS62e in control of shoot blight. In the first trial, EPS62e was applied at two concentrations, 10^8 cfu·mL^{-1} and 10^9 cfu·mL^{-1}. In the second trial, plants were treated only at 10^8 cfu·mL^{-1}, while, in the third trial, treatments were at 10^8 cfu·mL^{-1} and 10^7 cfu·mL^{-1}. Strain EPS62e was applied by spraying a suspension adjusted to the suitable concentration with an airbrush atomizer to run-off. Plants or detached branches were covered with plastic bags and incubated at 21 ºC, high relative humidity and 16 h of fluorescent light. After 24 h of incubation, plastic bags were removed and leaves were inoculated by deposition on the wounds of 10 μL of a pathogen suspension at 10^7 cfu·mL^{-1}. In both plant materials, two symmetrical wounds of 2 mm length were done in the reverse of the 3 youngest leaves of each shoot at the petiole base, approximately 1 cm of the beginning of the leaf. Inoculation of pathogen was done under biological safety conditions. Inoculated plants or detached branches were covered again with plastic bags and incubated at 21 ºC, high relative humidity and 16 h of fluorescent light.

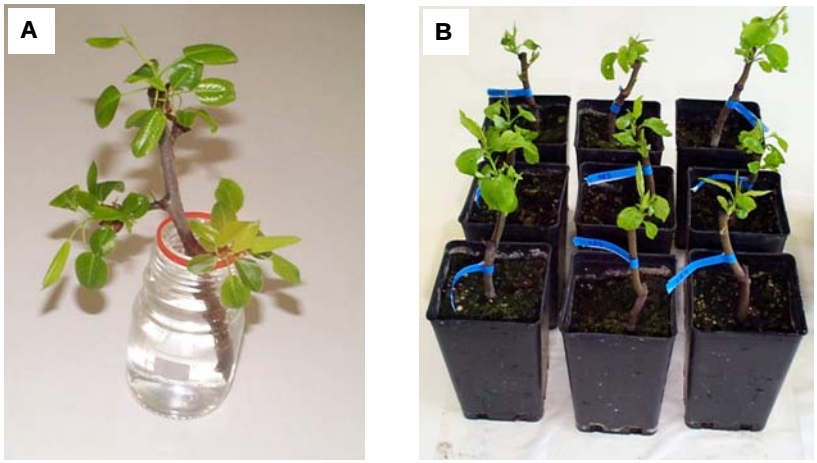

Figure 3.11. Young shoot of cultivar Doyenne du Comice obtained from detached branches containing vegetative buds forced to grow (**A**) and young shoots of cultivar Conference plants cultivated in pots (**B**).

The experimental design of the first trial consisted of three repetitions per treatment with three branches per repetition. Branches contained one or two shoots, and the three youngest leaves of each shoot were inoculated. In the second and third trial, the experimental design consisted of three repetitions per treatment of three plants per repetition. Each plant contained one or two shoots, and the three youngest leaves of each shoot were inoculated. Non-treated controls inoculated with water or with *E. amylovora* at 10^7 cfu·mL^{-1} were included in each trial.

Disease incidence and severity per repetition were evaluated after 10 days of pathogen inoculation and expressed using the equations described in the *section 4.1* from *Chapter 2*.

Statistical analysis

ANOVA was performed to test the effect of EPS62e treatments in the reduction of disease incidence and severity of shoot blight. The means were separated according to the Tukey's test at $P \leq 0.05$.

RESULTS

1. Build-up of a collection of antagonist isolates

During this study 169 isolates (*Annex 5*) come predominantly from leaves and roots of different rootstock samples (*Figure 3.12-A*) using the species oriented procedure. Most of the isolates presumably belonged to *P. fluorescens* and *E. herbicola* species according to phenotypic characteristics of growth on LB and KB agar (*Figure 3.12-B*).

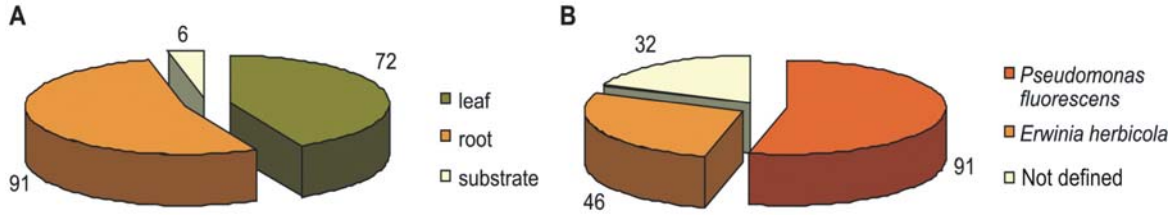

Figure 3.12. Number of isolates depending on the organ of origin (**A**) and bacterial species (**B**).

The complete collection according to *Table 3.1* was composed of 533 isolates obtained from different plant organs (leaf, fruit, root, bud, flower, rhizosphere) (*Figure 3.13-A*) and plants, mainly corresponding to the Rosaceous family. Besides, most of the isolates corresponded to bacterial isolates predominantly of *P. fluorescens* and *E. herbicola* species (*Figure 3.13-B*).

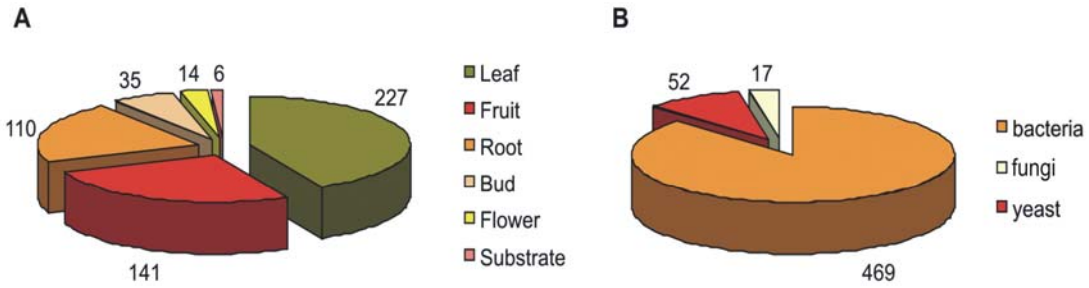

Figure 3.13. Number of isolates depending on the organ (**A**) and the microbial group (**B**) in the complete collection.

2. Screening of *E. amylovora* antagonists

Different levels of efficacy were observed among isolates in immature pear fruits (*Figure 3.14*). Isolates were grouped into five levels of efficacy of inhibition of *E. amylovora* infections: high (100-90%), moderate (90-70%), medium (70-40%), low (40-20%) and without significant activity (20-0%) (*Figure 3.15*) (*Annex 8*).

Figure 3.14. Infections caused by *E. amylovora* CUCM273 in immature pear fruits (cv. Passe Crassane) depending on the treatment by immersion with different putative antagonists. Fruits were treated at the corresponding concentration 24 h before inoculation with 10 µL of pathogen suspension at 10^7 cfu·mL^{-1}. in each wound. Fruits were incubated at 21ºC and 100% of relative humidity during 9 days.

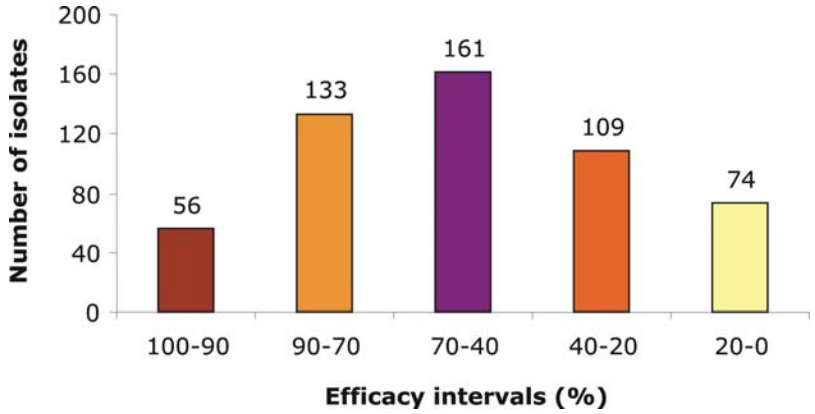

Figure 3.15. Distribution of efficacy of inhibition of infections among the 533 isolates in the immature pear fruit test. *E. amylovora* CUCM273 was inoculated at 10^7 cfu·mL^{-1}. Fruits were incubated during 9 days at 21 ºC and relative humidity of 100%.

Most of the isolates had certain efficacy in the inhibition of infections compared to the non-treated control, but only around a 10% of the screened isolates were very effective (>90%). A set of 61 isolates were retained for further analysis because they presented a high efficacy in the inhibition of infections.

3. Identification and characterization of a selection of potential BCAs

3.1. Identification

Sixty one isolates with high efficacy in inhibition of *E. amylovora* infections in immature pear fruits (*Annex 9*) were retained from the previous screening and were identified and characterized. Morphological criteria (Gram stain, motility), Oxidative/Fermentative test, oxidase test, and API strips were used as identification criteria. Isolates corresponding to *E. herbicola* and *S. marcescens* were Gram negative motile bacilli, aerobic facultative without oxidase activity that form orange or red colonies on LB, respectively. Isolates corresponding to *P. fluorescens* were Gram negative motile bacilli, strictly aerobic with oxidase activity that form fluorescent colonies on KB under UV light (366 nm). Identification was completed using API strips. API system 20E was used for isolates that presumably corresponded to *E. herbicola* and *S. marcescens*, and API system 20NE for *P. fluorescens* (*Tables 3.4, 3.5* and *3.6*). From the 61 isolates, 23 were identified as *E. herbicola*, 18 as *S. marcescens* and 20 as *P. fluorescens*.

Table 3.4. Characterization of *Erwinia herbicola* strains according to Oxidative/Fermentative test, oxidase test and API 20E system.

Strain	O	F	ONP	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	OX	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
EPS10e	+	+	+	-	-	-	+	-	-	+	-	+	-	-	+	+	-	-	+	+	+	+	+
EPS13e	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	+	+	+	+	+	+
EPS21e	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	-	+	+	+	+	+
EPS90e	+	-	+	-	-	-	+	-	-	+	-	+	-	-	+	+	-	-	+	+	+	+	+
EPS130e	+	+	+	-	-	-	+	-	-	+	-	+	-	-	+	+	-	+	+	+	+	+	+
EPS132e	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	-	+	+	+	+	+
EPS156e	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	-	+	+	-	+	+
EPS202e	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	-	+	+	-	+	+
EPS210e	+	+	+	-	-	-	+	-	-	+	-	+	-	-	+	+	-	-	+	+	+	+	+
EPS230e	+	+	+	-	-	-	+	-	-	+	-	+	-	-	+	+	-	+	+	+	+	+	+
EPS237e	+	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	-	+	+	+	+	+
EPS283e	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	-	+	+	+	+	+
EPS405	+	+	+	-	-	-	+	-	-	-	-	+	-	-	+	+	+	+	+	-	+	+	+
EPS412	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	+	+	+	+	+	+
EPS438	+	+	+	-	-	-	+	-	-	-	-	+	-	-	+	+	-	+	+	+	-	+	+
EPS455	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	-	+	+	+	+	+
EPS460	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+	-	+	+
EPS481	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+	+	+	-	+	+	+	+	+
EPS549	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	+	+	+	+	+	+
EPS594	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	-	+	+	+	+	+
EPS660	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	-	+	+	+	+	+
EPS697	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+	-	+	+
EPS708	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	+	-	+	+	+	+	+	+

O, glucose oxidation; F, glucose fermentation; ONP, β-galactosidase; ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; CIT, citrate use; H_2S, sulfide production; URE, urease; TDA, tryptophane deaminase; IND, indole production; VP, acetoin production, GEL; gelatinase; OX, ox idase; GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY and ARA, use of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose, respectively; +, positive result ;-, negative result.

Table 3.5. Characterization of *Serratia marcescens* strains according to Oxidative/Fermentative test, oxidase test and API 20E system.

Strain	O	F	ONP	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	OX	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
EPS259e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS301e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS311e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS313e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS317e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS318e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS321e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS326e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS327e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS330e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS342e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS343e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS345e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS348e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS349e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS352e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS357e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+
EPS358e	+	+	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+

O, glucose oxidation; F, glucose fermentation; ONP, β-galactosidase; ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; CIT, citrate use; H_2S, sulfide production; URE, urease; TDA, tryptophane deaminase; IND, indole production; VP, acetoin production, GEL; gelatinase; OX, ox idase; GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY and ARA, use of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose, respectively; +, positive result ;-, negative result.

Table 3.6. Characterization of *Pseudomonas fluorescens* strains according to Oxidative/Fermentative test, oxidase test and API 20NE system.

Strain	O	F	NO_3	TRP	GLU*	ADH	URE	ESC	GEL	PNG	OX	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC
EPS53e	+	-	-	-	-	+	-	-	+	-	+	+	-	+	+	-	-	+	+	-	+	+	-
EPS62e	+	-	-	-	-	+	-	-	+	-	+	+	+	-	+	-	-	+	+	-	+	+	-
EPS65e	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-
EPS82e	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	-
EPS87e	+	-	-	-	-	+	-	-	+	-	+	+	-	+	+	-	-	+	+	-	+	+	-
EPS89e	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	-	-	+	+	+	+	+	-
EPS95e	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	-
EPS102e	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-
EPS173e	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	-	-	+	+	+	+	+	-
EPS338	+	-	+	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-
EPS372	+	-	-	-	-	+	-	-	+	-	+	+	-	+	+	+	-	+	+	-	+	+	-
EPS538	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-
EPS664	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-
EPS684	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-
EPS702	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-
EPS720	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-
EPS734	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-
EPS735	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+	-
EPS784	+	-	-	-	-	+	-	-	+	-	+	+	+	-	+	+	-	+	-	+	-	+	-
EPS818	+	-	+	-	-	+	-	-	+	-	+	+	+	+	+	-	-	+	+	-	+	+	-

O, glucose oxidation; F, glucose fermentation; NO_3, reduction of nitrates to nitrites; TRP, indol production; GLU*, glucose fermentation; ADH, arginine dihydrolase; URE, urease; ESC, -glucosidase; GEL, gelatinase; PNG, β-galactosidase; OX, oxidase; GLU, ARA, MNE, MAN, NAG, MAL, GNT, CAP, ADI, MLT, CIT and PAC, assimilation of glucose, arabinose, manose, mannitol, N -acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenyl -acetate, respectively; +, positive result; -, negative result.

Aggregation analysis using Oxidative/Fermentative test, oxidase test, and API system resulted in dendrograms. These were obtained by means of the UPGMA method using the similarity index calculated by the simple matching coefficient (*Figures 3.16* and *3.17*). The dendrogram corresponding to *S. marcescens* was not made because differences between strains were not observed using the set of tests selected.

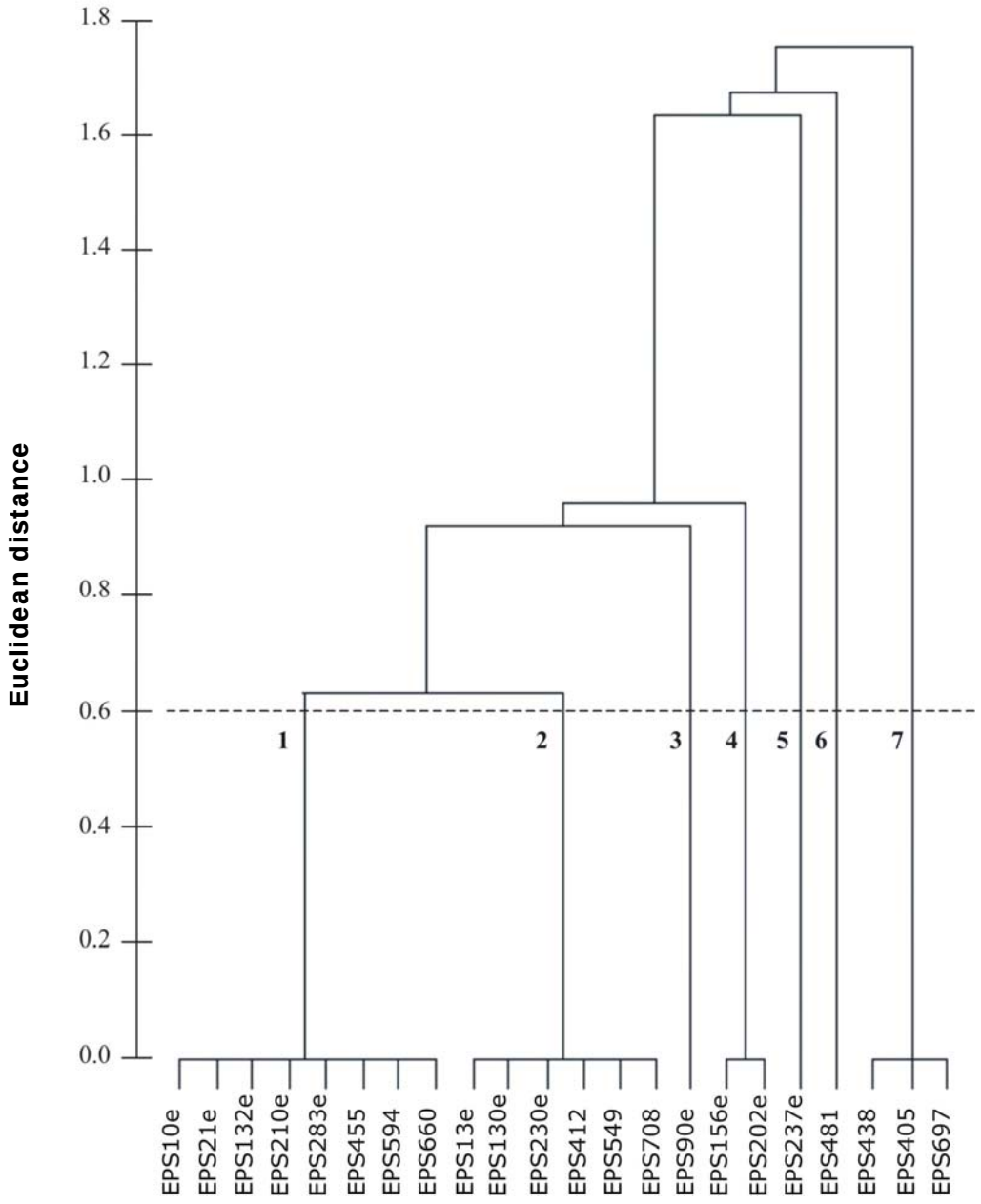

Figure 3.16. Dendrogram of the relatedness between the 23 strains of *E. herbicola* according to oxidative/fermentative test, oxidase test and API system. The similarity matrix between bacterial isolates was calculated using the Euclidean distance. Cluster analysis was performed by applying the Agglomerative Hierarchical group method through cluster analysis procedure of S-PLUS package. Dotted line indicates the Euclidean distance to obtain the groups described in *Table 3.7*.

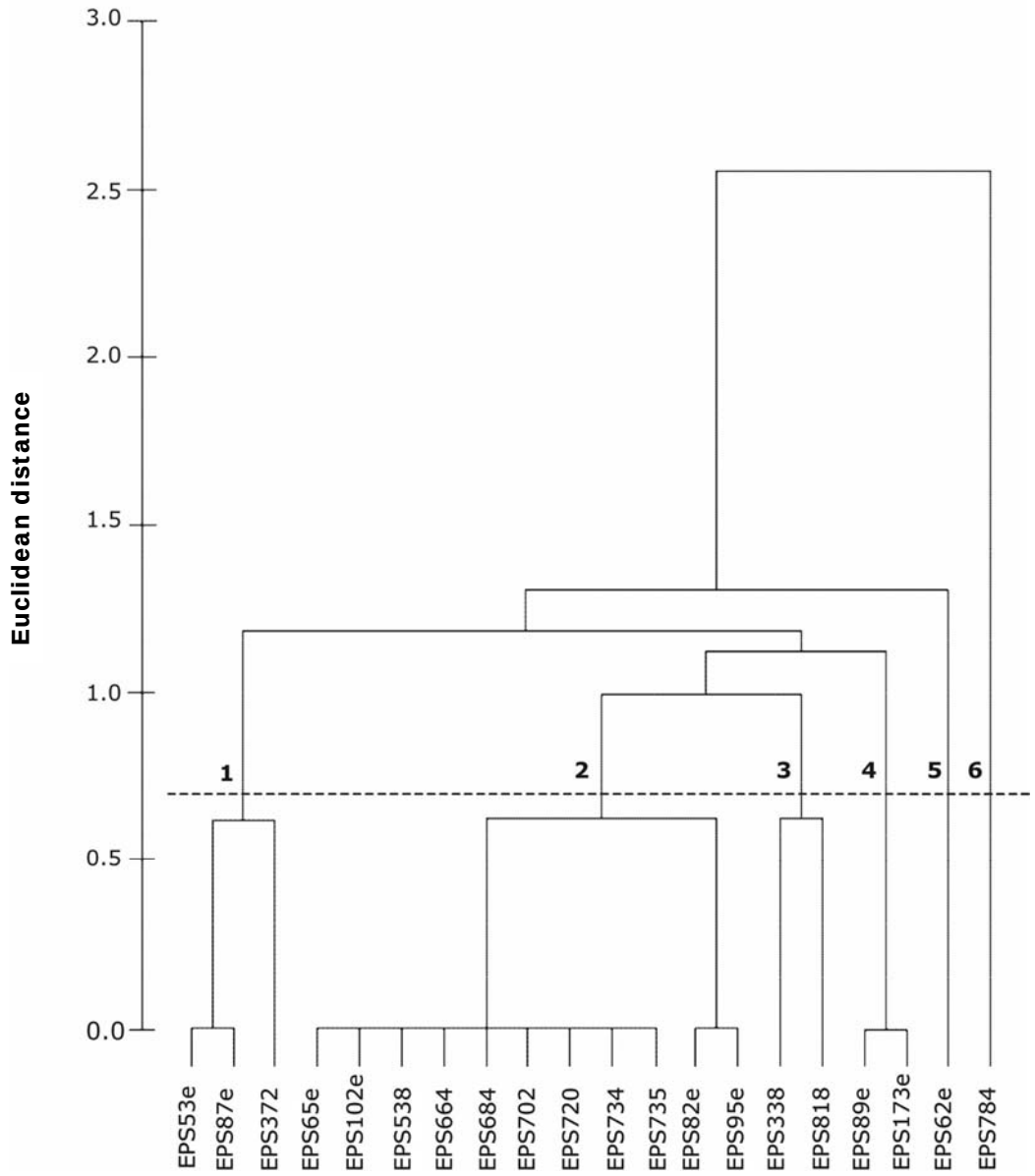

Figure 3.17. Dendrogram of the relatedness between the 20 strains of *P. fluorescens* according to oxidative/fermentative test, oxidase test and API system. The similarity matrix between bacterial isolates was calculated using the Euclidean distance. Cluster analysis was performed by applying the Agglomerative Hierarchical group method through cluster analysis procedure of S-PLUS package. Dotted line indicates the Euclidean distance to obtain the groups described in *Table 3.9.*

E. herbicola strains were separated to a Euclidean distance of 0.6 obtaining 7 groups (*Table 3.7*). Only 8 out of 23 tests gave differential response among strains including glucose fermentation, citrate use, tryptophane deaminase, indole production, acetoin production, and use of mannitol, inositol, and sucrose (*Table 3.8*). The more variable test was citrate that was positive for groups four and five, variable for groups one, two and seven, and negative for groups three and six. The strains that differed more were EPS237e, EPS481, EPS438, EPS405 and EPS697 that corresponded to the strains of groups five, six and seven.

Table 3.7. Grouping of *E. herbicola* strains situated at an average distance of 0.6 according to oxidative/fermentative test, oxidase test and API system 20E

Group	Strains
1	10e 132e 21e 210e 283e 455 594 660
2	13e 130e 230 412 549 708
3	90e
4	156e 202e
5	237e
6	481
7	438 460 697

Table 3.8. Differential characteristics based on oxidative/fermentative test, oxidase test and API system 20E for each group of *E. herbicola* isolates indicated in *Table 3.7*

Group	F	CIT	TDA	IND	VP	MAN	INO	SAC
1	+	d	+	-	+	-	-	+
2	+	d	+	-	+	-	+	+
3	+	-	+	-	+	-	-	-
4	-	+	+	-	+	-	-	+
5	+	+	+	+	-	-	-	+
6	+	-	-	-	+	+	-	+
7	+	d	-	-	+	-	+	-

F, glucose fermentation; CIT, citrate use; TDA, tryptophane deaminase; IND, indole production; VP, acetoin production; MAN, INO, and SAC, use of mannitol, inositol, and sucrose, respectively; +, all the strains are positive; -, all the strains are negative; d, variable result.

P. fluorescens strains were separated to a Euclidean distance of 0.7 obtaining 6 groups (*Table 3.9*). Only 7 out of 23 tests performed gave differential response among the six groups of strains including reduction of nitrates to nitrites, assimilation of glucose, arabinose, mannitol, gluconate, caprate and adipate (*Table 3.10*). The most variable tests were manose and caprate assimilation. Manose assimilation was positive for groups two and six, variable for one and four, and negative for three and five. Caprate assimilation was positive for groups three and six, variable for two, and negative for one, four and five. The strains that differed more from the others were EPS627e and EPS784 that corresponded to strains of groups five and six, respectively.

Table 3.9. Grouping of *P. fluorescens* strains obtained at an average distance of 0.7 according to oxidative/fermentative test, oxidase test and API system 20NE

Group	Strains
1	EPS372 EPS53e EPS87e
2	EPS102e EPS538 EPS65e EPS664 EPS684 EPS702 EPS720 EPS734 EPS735 EPS82e EPS95e
3	EPS338 EPS818
4	EPS173e EPS89e
5	EPS62e
6	EPS784

Table 3.10. Differential characteristics based on oxidative/fermentative test, oxidase test and API system 20NE for each group of *P. fluorescens* isolates indicated in *Table 3.9*

Group	NO_3	GLU	ARA	MAN	GNT	CAP	ADI
1	-	-	+	d	+	-	+
2	-	+	+	+	+	d	+
3	-	+	+	-	+	+	+
4	+	+	+	d	+	-	+
5	-	+	-	-	+	-	+
6	-	+	-	+	-	+	-

NO_3, reduction of nitrates to nitrites; GLU, ARA, MAN, GNT, CAP and ADI, assimilation of glucose, arabinose, mannitol, gluconate, caprate and adipate, respectively; +, all the strains are positive; -, all the strains are negative; d, variable result.

3.2. Characterization of the antagonistic and biocontrol capacity of the strains

The 61 strains showing high efficacy in the immature pear fruits assay (*Annex 9*) were characterized according to their antagonism on agar media against different plant pathogens, capacity to produce several antimicrobial compounds, and efficacy in inhibition of *E. amylovora* infections in immature pear fruits.

The antagonism spectrum of the selected strains was assessed against several plant pathogens (*E. amylovora*, *P. syringae*, *X. campestris* and *P. expansum*) on different culture media (LB, KB, KB-fe, GA, GA-fe and PDA) (*Figure 3.18*). In addition, strains were characterized for their capacity to produce several compounds with biocontrol activity (antibiotics, cyanide, chitinases, siderophores, indolacetic acid) (*Figures 3.19* and *3.20*) and the efficacy in inhibition of *E. amylovora* infections in immature pear fruits (*Tables 3.11, 3.12* and *3.13*). Because *X. campestris* only grew on LB medium, no results were reported for this species in other tested media.

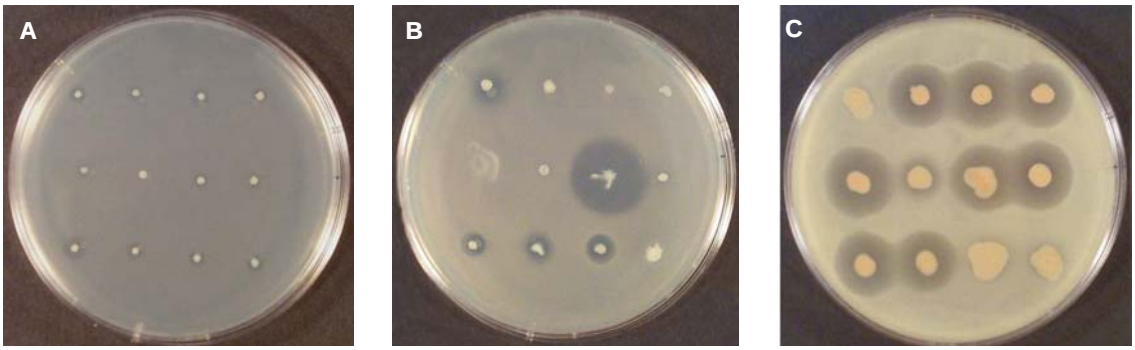

Figure 3.18. Antagonism of several bacterial strains against (**A**): *Pseudomonas syringae* on GA; (**B**): *E. amylovora* on GA; and (**C**): *P. syringae* on LB.

Table 3.11. Characterization of *E. herbicola* strains according to the efficacy in the inhibition of *E. amylovora* infections in immature pear fruits, production of microbial compounds, and *in vitro* antagonism against several plant pathogens on different media.

									***In vitro* antagonism against**										
		Production of							*P. syringae*			*X. campestris*	*E. amylovora*			*P. expansum*		
Strain	**EBCEa** (%)	**HCN**	**Sid**	**Chit**	**Phl**	**PCA**	**Prn**	**IAA**	**LB**	**GA**	**GA-fe**	**LB**	**LB**	**GA**	**GA-fe**	**LB**	**GA**	**PDA**
EPS10e	22.22	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
EPS13e	27.78	-	+	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-
EPS21e	50.00	-	+	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-
EPS90e	44.44	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
EPS130e	33.33	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
EPS132e	38.89	-	+	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-
EPS156e	55.56	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
EPS202e	50.00	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS210e	50.00	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS230e	33.33	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS237e	63.89	-	+	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-
EPS283e	55.56	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
EPS405	27.78	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
EPS412	36.11	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-
EPS438	55.56	-	+	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-
EPS455	11.11	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
EPS460	47.22	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-
EPS481	25.00	-	+	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-
EPS549	36.11	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-
EPS594	19.44	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
EPS660	38.89	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS697	41.67	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS708	38.89	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

EBCEa, efficacy in the inhibition of *E. amylovora* infections in immature pear fruits; HCN, cyanide; Sid, siderophore; Chit, chitinases; Phl, 2,4-diacetylphloroglucinol; PCA, phenazine-1-carboxylic; Prn, pyrrolnitrin; IAA, Indolacetic acid; LB, Luria Bertani medium; GA, glucose-asparagine medium; GA-fe, glucose-asparagine medium amended with $FeCl_3$ 50 μM; PDA, potato dextrose agar; +, positive; -, negative.

Table 3.12. Characterization of *S. marcescens* strains according to the efficacy in the inhibition of *E. amylovora* infections in immature pear fruits, production of microbial compounds, and *in vitro* antagonism against several plant pathogens on different media.

									***In vitro* antagonism against**									
		Production of							*P. syringae*			*X. campestris*	*E. amylovora*			*P. expansum*		
Strain	**EBCEa** (%)	**HCN**	**Sid**	**Chit**	**Phl**	**PCA**	**Prn**	**IAA**	**LB**	**GA**	**GA-fe**	**LB**	**LB**	**GA**	**GA-fe**	**LB**	**GA**	**PDA**
EPS259e	11.11	-	+	+	-	-	-	-	-	+	+	-	-	-	-	+	+	+
EPS301e	16.67	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS311e	36.11	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS313e	30.56	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS317e	19.44	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS318e	0.00	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS321e	25.00	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS326e	11.11	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS327e	11.11	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS330e	19.44	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS342e	2.78	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS343e	27.78	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS345e	5.56	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS348e	33.33	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS349e	36.11	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS352e	16.67	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS357e	33.33	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+
EPS358e	0.00	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+

EBCEa, efficacy in the inhibition of *E. amylovora* infections in immature pear fruits; HCN, cyanide; Sid, siderophore; Chit, chitinases; Phl, 2,4-diacetylphloroglucinol ; PCA, phenazine-1-carboxylic; Prn, pyrrolnitrin; IAA, Indolacetic acid; LB, Luria Bertani medium; GA, glucose-asparagine medium; GA-fe, glucose-asparagine medium amended with $FeCl_3$ 50 μM; PDA, potato dextrose agar; +, positive; -, negative.

Table 3.13. Characterization of *P. fluorescens* strains according to the efficacy in the inhibition of *E. amylovora* infections in immature pear fruits, production of microbial compounds, and *in vitro* antagonism against several plant pathogens on different media.

									***In vitro* antagonism against**									
		Production of							*P. syringae*			*X. campestris*	*E. amylovora*			*P. expansum*		
Strain	**EBCEa** (%)	**HCN**	**Sid**	**Chit**	**Phl**	**PCA**	**Prn**	**IAA**	**LB**	**GA**	**GA-fe**	**LB**	**LB**	**GA**	**GA-fe**	**LB**	**GA**	**PDA**
EPS53e	25.00	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
EPS62e	58.33	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS65e	33.33	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
EPS82e	52.78	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
EPS87e	33.33	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
EPS89e	11.11	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
EPS95e	41.67	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS102e	30.56	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
EPS173e	30.56	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS338	33.33	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS372	58.33	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+
EPS538	52.78	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
EPS664	16.67	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS684	63.89	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS702	5.56	-	+	-	-	-	-	-	-	-	+	-	-	+	+	+	+	-
EPS720	16.67	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPS734	55.56	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
EPS735	11.11	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+
EPS784	36.11	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
EPS818	16.67	-	+	-	-	-	-	-	+	+	+	-	-	-	-	+	+	+

EBCEa, efficacy in the inhibition of *E. amylovora* infections in immature pear fruits; HCN, cyanide; Sid, siderophore; Chit, chitinases; Phl, 2,4-diacetylphloroglucinol ; PCA, phenazine-1-carboxylic; Prn, pyrrolnitrin; IAA, Indolacetic acid; LB, Luria Bertani medium; GA, glucose-asparagine medium; GA -fe, glucose-asparagine medium amended with $FeCl_3$ 50 μM; PDA, potato dextrose agar; +, positive; -, negative.

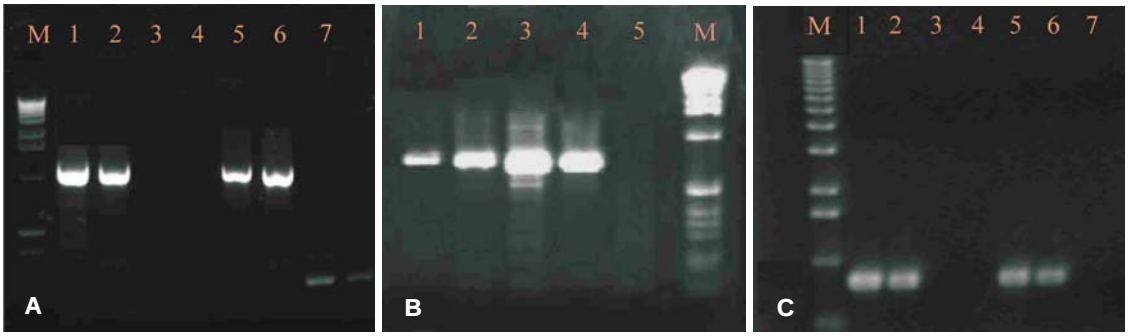

Figure 3.19. Agarose gel electrophoresis of the PCR products amplified with primers (**A**): Phl2a and Phl2b. Lane M, 1 Kb DNA ladder; lane 1-6, Several strains; lane 7, Negative control. (**B**): PCA2a and PCA3b. Lane M, 1 Kb DNA ladder; lane 1-4, Several strains; lane 5, Negative control. (**C**): Prna and Prnb. Lane M, 1 Kb DNA ladder; lane 1-6, several strains; lane 7, Negative control.

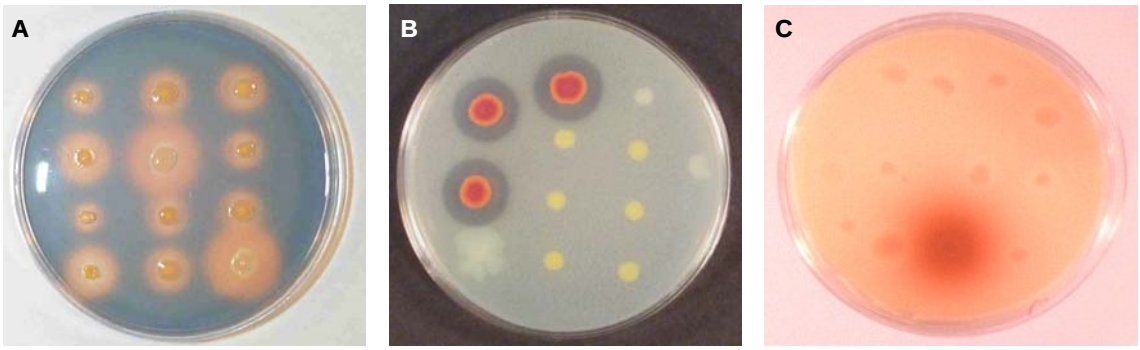

Figure 3.20. Siderophore production on CAS agar (**A**), chitinase production on colloidal chitin agar (**B**), and IAA production on LB agar amended with tryptophan (**C**).

Finally, selected strains were retested for their efficacy in the inhibition of *E. amylovora* infections in immature pear fruits to verify the consistence of their efficacy. Significant effect of treatments with strains in the inhibition of *E. amylovora* infections was found ($F=3.60$, $P<0.001$). Some strains lost their efficacy in the second trial. Besides, it was observed a global decrease of the efficacy in all treatments. Thus, the maximum efficacy observed in the second trial was 65% instead 100% of observed in the first trial.

The treatments were grouped using the Waller-Duncan's mean separation test in order to get the strains that have the highest efficacy and consistency in the immature pear fruits assay. Strains sorted according to their efficacy are shown in the *Table 3.14*.

Table 3.14. Efficacy of the 61 selected bacterial strains in the inhibition of infections of *E. amylovora* EPS101 in immature pear fruits

Strain	Species	Efficacy[x] (%)		Strain	Species	Efficacy (%)	
EPS684	*P. fluorescens*	64	A	EPS348e	*S. marcescens*	33	BCDEFGHI
EPS237e	*E. herbicola*	64	A	EPS173e	*P. fluorescens*	31	CDEFGHIJ
EPS62e	*P. fluorescens*	58	AB	EPS313e	*S. marcescens*	31	CDEFGHIJ
EPS156e	*E. herbicola*	56	ABC	EPS102e	*P. fluorescens*	31	CDEFGHIJ
EPS734	*P. fluorescens*	56	ABC	EPS13e	*E. herbicola*	28	DEFGHIJK
EPS283e	*E. herbicola*	56	ABC	EPS405	*E. herbicola*	28	DEFGHIJK
EPS438	*E. herbicola*	56	ABC	EPS343e	*S. marcescens*	28	DEFGHIJK
EPS372	*P. fluorescens*	53	ABCD	EPS481	*E. herbicola*	25	EFGHIJKL
EPS538	*P. fluorescens*	53	ABCD	EPS321e	*S. marcescens*	25	EFGHIJKL
EPS82e	*P. fluorescens*	53	ABCD	EPS53e	*P. fluorescens*	25	EFGHIJKL
EPS210e	*E. herbicola*	50	ABCDE	EPS10e	*E. herbicola*	22	FGHIJKL
EPS202e	*E. herbicola*	50	ABCDE	EPS594	*E. herbicola*	19	GHIJKL
EPS21e	*E. herbicola*	50	ABCDE	EPS330e	*S. marcescens*	19	GHIJKL
EPS460	*E. herbicola*	47	ABCDEF	EPS317e	*S. marcescens*	19	GHIJKL
EPS90e	*E. herbicola*	44	ABCDEFG	EPS301e	*S. marcescens*	17	HIJKL
EPS95e	*E. herbicola*	42	ABCDEFGH	EPS352e	*S. marcescens*	17	HIJKL
EPS697	*E. herbicola*	42	ABCDEFGH	EPS818	*P. fluorescens*	17	HIJKL
EPS132e	*E. herbicola*	39	ABCDEFGH	EPS720	*P. fluorescens*	17	HIJKL
EPS708	*E. herbicola*	39	ABCDEFGH	EPS664	*P. fluorescens*	17	HIJKL
EPS660	*P. fluorescens*	39	ABCDEFGH	EPS455	*E. herbicola*	11	IJKL
EPS549	*E. herbicola*	36	BCDEFGHI	EPS326e	*S. marcescens*	11	IJKL
EPS784	*P. fluorescens*	36	BCDEFGHI	EPS259e	*S. marcescens*	11	IJKL
EPS412	*E. herbicola*	36	BCDEFGHI	EPS735	*P. fluorescens*	11	IJKL
EPS349e	*S. marcescens*	36	BCDEFGHI	EPS89e	*E. herbicola*	11	IJKL
EPS311e	*S. marcescens*	36	BCDEFGHI	EPS327e	*S. marcescens*	11	IJKL
EPS357e	*S. marcescens*	33	BCDEFGHI	EPS345e	*S. marcescens*	6	JKL
EPS230	*E. herbicola*	33	BCDEFGHI	EPS702	*P. fluorescens*	6	JKL
EPS338	*E. herbicola*	33	BCDEFGHI	EPS342e	*S. marcescens*	3	KL
EPS65e	*P. fluorescens*	33	BCDEFGHI	EPS318e	*S. marcescens*	0	L
EPS87e	*P. fluorescens*	33	BCDEFGHI	EPS358e	*S. marcescens*	0	L
EPS130e	*E. herbicola*	33	BCDEFGHI				

[x]Immature pear fruits (cv. Passe Crassane) were treated by immersion with isolate suspensions at 10^8 cfu·mL^{-1} and inoculated with *E. amylovora* at 10^7 cfu·mL^{-1} after 24 h of treatments. The fruits were incubated at 21ºC and high relative humidity. Efficacy was assessed after 9 days of pathogen inoculation according to the incidence observed in the non-treated control (100%). Means with the same letter are not significantly different (P>0.05) according to Waller-Duncan's test.
Color gradation indicates degree of efficacy; dark gray, high efficacy; soft gray, moderate efficacy; pale gray, low efficacy.

Apart from intensity of infections at 9 days from the pathogen inoculation, the progress of disease through time was recorded. Disease progress curves were different depending on the strain (*Figure 3.21*) and differences were more evident in an advanced stage of the infection process. The incidence of infections in the non-treated control attained values of 100% after 9 days of the pathogen inoculation, while in some treatments, such as EPS62e and EPS372, the levels of incidence did not reached values above 50%.

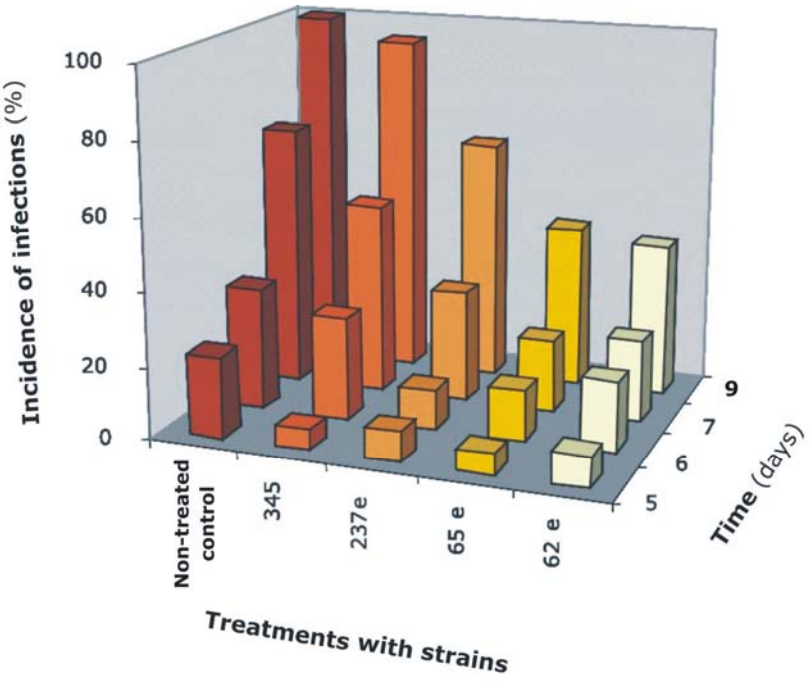

Figure 3.21. Infection progress curves of *E. amylovora* in immature pear fruits depending on biocontrol treatment. Treatments were based on the application of putative antagonists to wounded fruits by immersion in suspensions at 10^8 cfu·mL^{-1}. A non-treated control only inoculated with *E. amylovora* was included. Incubation was performed along 10 days in controlled conditions at 21 ºC, high relative humidity and 16 h of light.

Only a 16% of strains tested against EPS101 (data correspond to second trial performed in immature pear fruits) maintained a high efficacy (>50%) in the inhibition of infections. Eight strains were selected as potential biocontrol agents corresponding to only a 1.5% of the 533 isolates tested. However, in spite of their high efficacy strains EPS372 and EPS237 were discarded because they were positive for HR test in tobacco and for INA.

Aggregation analysis was performed for the 61 strains based on the spectrum of antagonism, efficacy in immature pear fruits and capacity to produce antimicrobial compounds. A dendrogram using the Euclidean distance was obtained by means of the UPGMA method (*Figure 3.22*).

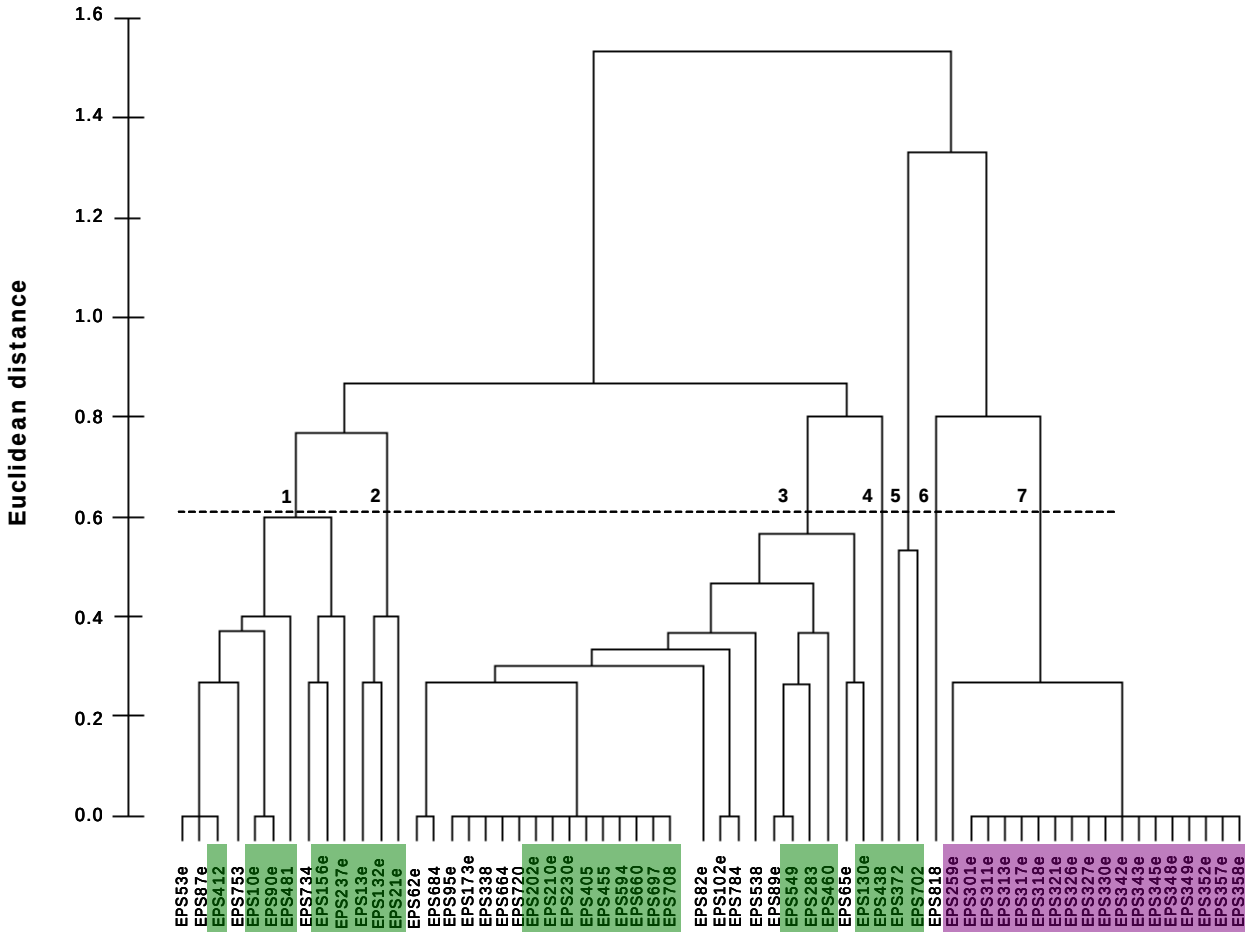

Figure 3.22. Dendrogram of the relatedness between the 61 antagonistic strains using the variables of production of antimicrobial compounds, efficacy in the inhibition of infections caused by *E. amylovora* in immature pear fruits and antagonism against several pathogens on different culture media. The similarity matrix between bacterial isolates was calculated using the Euclidean distance. Cluster analysis was performed by applying the Agglomerative Hierarchical group method through cluster analysis procedure of S-PLUS. Dotted line indicates the Euclidean distance to obtain the groups described in *Table 3.15*. White, green and purple marks correspond to the strains of *P. fluorescens*, *E. herbicola* and *S. marcescens*, respectively.

Strains of *E. herbicola*, *P. fluorescens* and *S. marcescens* were separated at Euclidean distance of 0.64 and seven groups were obtained (*Table 3.15*). Strains were grouped according to the differential results on the production of several antimicrobial compounds, the efficacy in immature pear fruit test and the spectrum of *in vitro* antagonism against several plant pathogens on different culture media (*Table 3.16*).

Table 3.15. Groups of strains situated at a Euclidean distance of 0.64 according to the production of several compounds, efficacy in the inhibition of infections caused by *E. amylovora* in immature pear fruits and the spectrum of antagonism against several plant pathogens on different culture media. Marked strains in green corresponded to *E. herbicola* species, in purple to *S. marcescens* and without mark to *P. fluorescens.* White, green and purple marks correspond to the strains of *P. fluorescens*, *E. herbicola* and *S. marcescens*, respectively.

Group	Strains
1	EPS10e EPS156e EPS237e EPS412 EPS481 EPS53e EPS734 EPS735 EPS87e EPS90e
2	EPS13e EPS132e EPS21e
3	EPS102e EPS130e EPS173e EPS202e EPS210e EPS230e EPS283e EPS338 EPS405 EPS455 EPS460 EPS538 EPS549 EPS594 EPS62e EPS65e EPS660 EPS664 EPS684 EPS697 EPS708 EPS720 EPS784 EPS82e EPS89e EPS95e
4	EPS438
5	EPS372 EPS702
6	EPS818
7	EPS259e EPS301e EPS311e EPS313e EPS317e EPS318e EPS321e EPS326e EPS327e EPS330e EPS342e EPS343e EPS345e EPS348e EPS349e EPS352e EPS357e EPS358e

Taula 3.16. Differential characteristics of the groups from *Table 3.15* obtained by cluster analysis

						Antagonism against							
		Production of				*P. syringae*			*E. amylovora*			*P. expansum*	
Group	**EIEa**	**HCN**	**Chit**	**Sid**	**IAA**	**LB**	**GA**	**GA-fe**	**GA**	**GA-fe**	**LB**	**GA**	**PDA**
1	d	d	d	d	d	d	d	-	+	d	-	-	d
2	l	-	-	+	-	-	d	+	d	+	-	-	-
3	d	d	-	+	d	d	d	d	-	-	d	-	d
4	h	-	-	+	+	-	+	+	-	-	-	-	-
5	d	-	-	+	-	-	-	+	+	d	+	+	d
6	d	-	-	+	-	+	+	+	-	-	+	+	+
7	l	-	+	+	-	-	+	d	-	-	+	+	+

EIEa, efficacy in the inhibition of *E. amylovora* infections in immature pear fruits; HCN, cyanide; Chit, chitinases; Sid, siderophores; IAA, indolacetic acid; LB, Luria Bertani medium; GA, gluose-asparagine medium; GA-fe, glucose-asparagine medium amended with $FeCl_3$ 50 μM; PDA, potato dextrose agar; l, low efficacy; h, high efficacy; +, all the strains are positive; -, all the strains are negative; d, variable result.

None of the 61 strains produced the antibiotics Phl, PCA or Prn. In contrast, all the strains produced siderophores, except *E. herbicola* EPS10e (group 1). Besides, all the strains of *S. marcescens* produced chitinases (group 6), while none of *E. herbicola* strains and only *P. fluorescens* EPS734 and EPS735 (group 1) were chitinase producers. Finally, only *P.*

fluorescens EPS65e (group 3) and EPS735 (group 1) produced HCN and *E. herbicola* EPS412 (group 1), EPS405, EPS455, EPS594, EPS549 (group 3) and EPS438 (group 4) produced IAA.

None of the strains inhibited *X. campestris* and *E. amylovora* on LB medium. Most of groups of strains developed antagonism against *P. syringae* on GA and GA-fe except the groups five and one, respectively. In contrast, only groups one, two and five contained strains that inhibited *E. amylovora in vitro*. The antagonist strains against *P. expansum* were predominantly distributed in groups five, six and seven.

Strains with high efficacy in the immature pear fruit test were distributed along the groups, and only group four presented high efficacy due to the fact that was composed of one strain. Although, groups two and seven predominantly included strains with low efficacy.

In relation to the distribution of strains according to the species to which pertain, most of the groups presented a variable composition of *P. fluorescens* and *E. herbicola* strains, except the groups two and five, composed of *E. herbicola* strains, six, composed of *P. fluorescens* strains, and group seven that included all the *S. marcescens* strains.

Associations among the traits of the strains were studied by correspondence analysis of these variables with their corresponding categories. Two dimensions that explain the 43% of total variance were obtained. The first dimension explains 31% of the variance whereas the second dimension only 11%. The projection of the different categories for each variable in the plane described for the two dimensions are shown in *Figure 3.23*.

The first dimension is very associated to the antagonism. Antagonism against *P. expansum* (*ape*) is located in the upper part of the axis, while the antagonism against *P. syringae* (*aps*) and *E. amylovora* (*aea*) and the non antagonism against *P. expansum* are located in the lower part. Antagonism is also related with the variable production of inhibitory compounds. Antagonism against *P. expansum* is associated to *S. marcescens* (*Sm*) and chitinase production (*Chit*), while antagonism against bacteria is associated to *P. fluorescens* (*Pf*) and cyanide production (*H*). A relation between high efficacy in inhibition of infections by *E. amylovora* in immature pear fruits (*Hef*) and antagonism against bacteria was also found for the *P. fluorescens* strains.

The second dimension is also associated with antagonism and less with the species. Thus the absence of antagonism against bacteria associated with *E. herbicola* is located in the left of the axis, whereas the corresponding antagonism associated to *P. fluorescens* is located in the right side. Association with the plant organ and the plant host is observed along the second dimension. The strains isolated from the rhizosphere are associated to *E. herbicola* and are located in the left side, while strains isolated from the phyllosphere are more related to *P. fluorescens* and *S. marcescens* and are located in the right side.

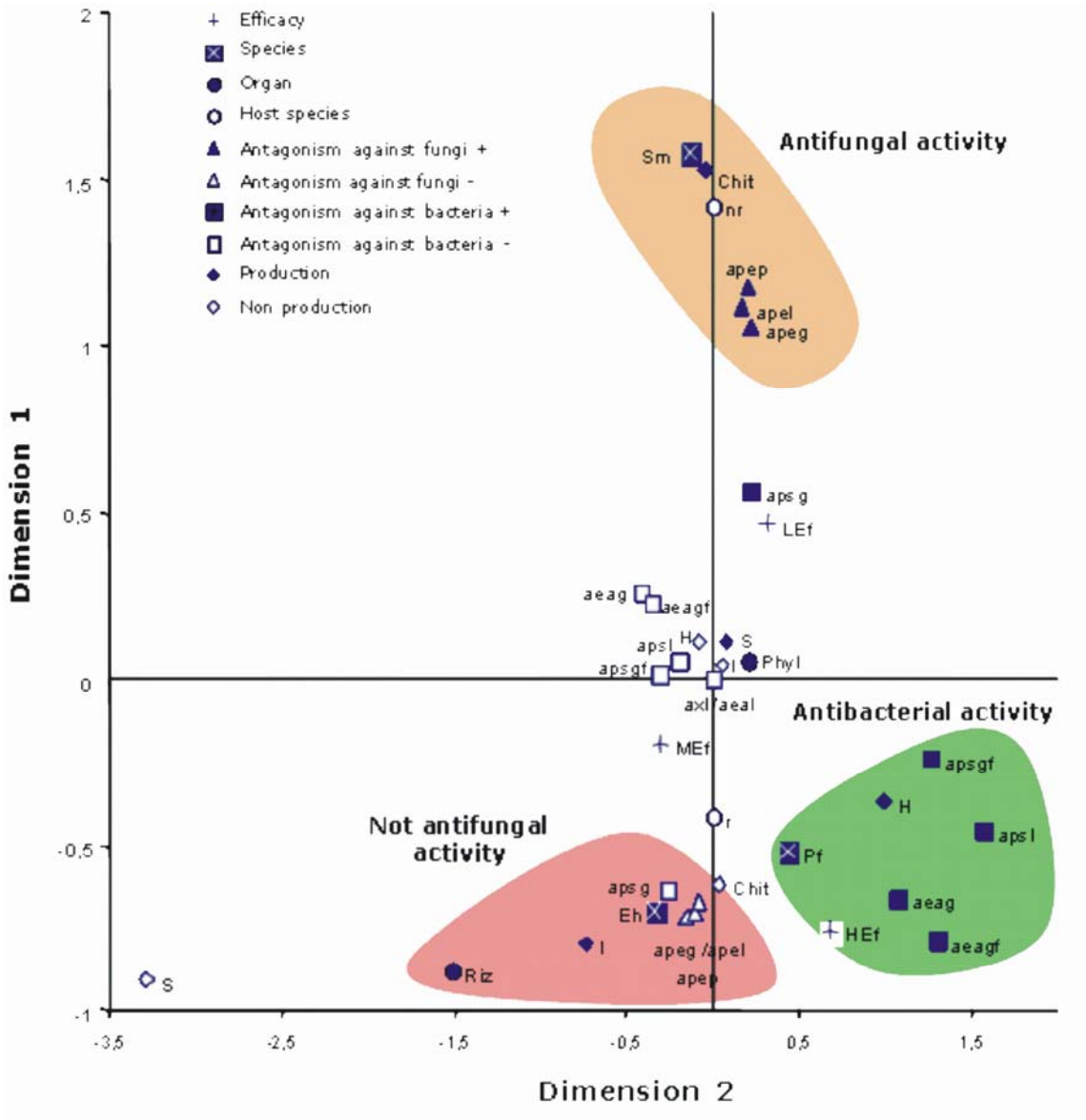

Figure 3.23. Projection of the variables describing the 61 strains (species, host species, source organ, antagonism against several pathogens on different culture media, production of several biocontrol compounds, and efficacy in the inhibition of infections by *E. amylovora* in immature pear fruits) in the plane described by the two dimensions that explain a 43% of variance. Symbols: Eh, *E. herbicola*; Ps, *P. fluorescens*; Sm, *S. marcescens*; r, rosaceus plants; nr, non rosaceus plants; Riz, rizosphere; Phyl, phyllosfere; apep/apeg/apel, antagonism against *P. expansum* on PDA, GA and LB respectively; apsg/apsgf/apsl, antagonism against *P. syringae* on GA, GA-fe and LB respectively; aeag/aeagf/aeal, antagonism against *E. amylovora* on GA, GA-fe and LB respectively, axl, antagonism against *X. campestris* on LB; H, cyanide; I, Indolacetic acid; Chit, Chitinases; S, siderophores; LEf/MEf/HEf, low, moderate and high efficacy in the inhibition of infections by *E. amylovora* in immature pear fruits, respectively.

4. Efficacy assays and selection of a biological control agent of fire blight

4.1. Detached pear flowers assays

The eight more effective strains which inhibited *E. amylovora* infections in the immature pear fruit assay (EPS62e, EPS156e, EPS283e, EPS438, EPS460, EPS538, EPS684, and EPS734) were tested for their ability to suppress blossom blight (*Figures 3.24*). Two trials were performed.

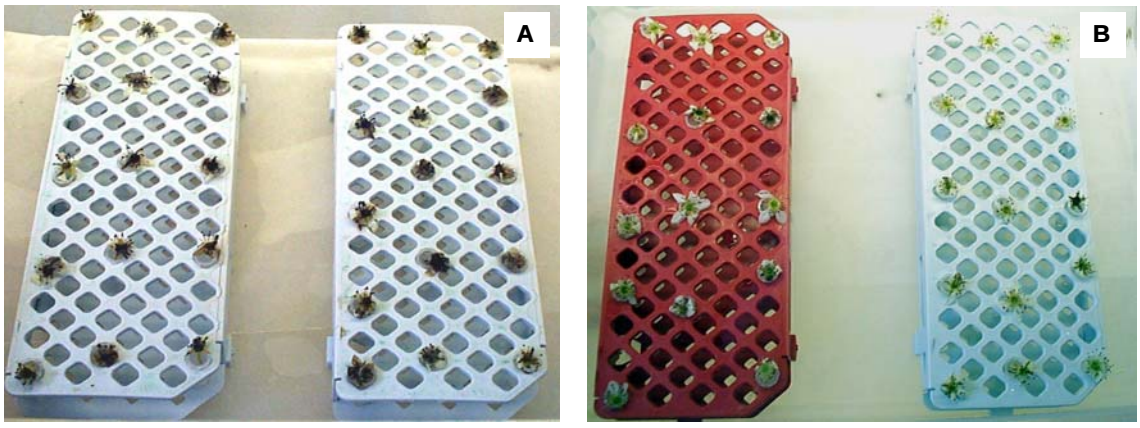

Figure 3.24. Pear flowers (cv. Doyenne du Comice) corresponding to the pathogen inoculated non-treated controls (**A**) and treated with a suspension of strain EPS62e (**B**). The flowers were inoculated with *E. amylovora* EPS101 at 10^7 cfu·mL^{-1} after 24 h of treatment by spraying with a EPS62e suspension at 10^8 cfu·mL^{-1}. Flowers were incubated during 8 days at 21 ºC, high relative humidity and photoperiod with 16 h of light.

In the first trial, the eight strains were tested at the optimum pathogen concentration of 10^7 cfu·mL^{-1}. Only strains EPS283e, EPS684, EPS734 and EPS62e showed significant control of blossom blight after 8 days of pathogen inoculation ($P<0.001$) (*Figure 3.25*). Strain EPS62e was the most effective with an efficacy of 83% both in the reduction of the incidence and of the severity of infections. The *E. herbicola* strains EPS283e, EPS156e, EPS460 and EPS438, were less effective (0 to 30%). Besides, *P. fluorescens* EPS538 produced a smooth necrosis in flowers 24 h after the treatment, and for this reason the strain was discarded.

In the second trial, the four most effective strains according to the first trial (EPS283e, EPS734, EPS684 and EPS62e) were retested to evaluate their consistence in blossom blight control. Only strains EPS734 and EPS62e maintained significant reduction of incidence and severity of blossom blight. *P. fluorescens* EPS62e was confirmed as the most effective strain with an efficacy of 65% in the reduction of severity and of 60% in the reduction of incidence of blossom blight (*Figure 3.26*).

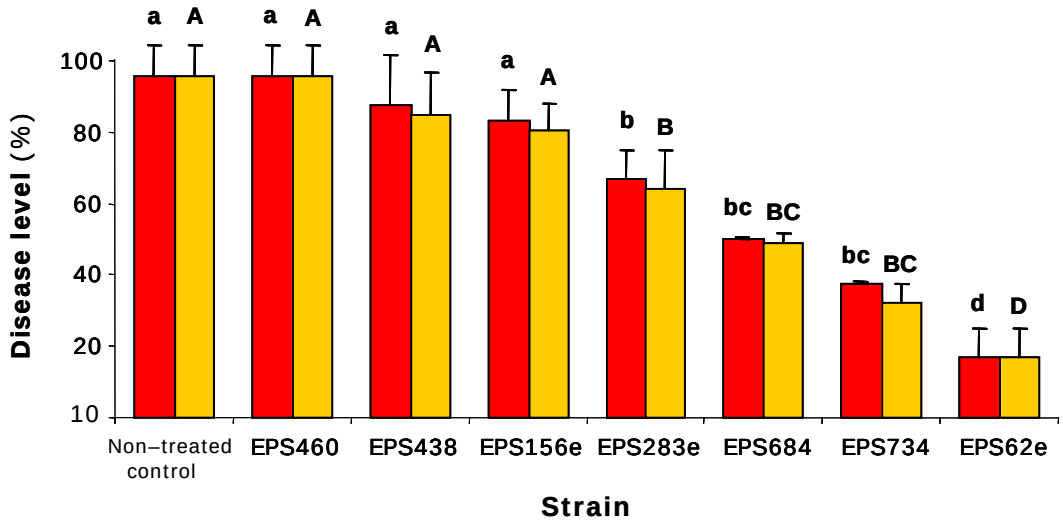

Figure 3.25. Effect of treatments with different antagonists on disease incidence (red series) and severity (yellow series) of infections in Doyenne du Comice pear flowers inoculated with *E. amylovora*. Data correspond to the first trial. Flowers were treated by spraying with the antagonist suspension at 10^8 cfu·mL^{-1} and inoculated 24 h later by deposition of 10 µl of a pathogen suspension at 10^7 cfu·mL^{-1}. Incidence and severity were assessed after 8 days at 21 ºC and high relative humidity. The same (capital and non capital) letter indicates that significant differences do not exist (P>0.05) according to Tukey's test.

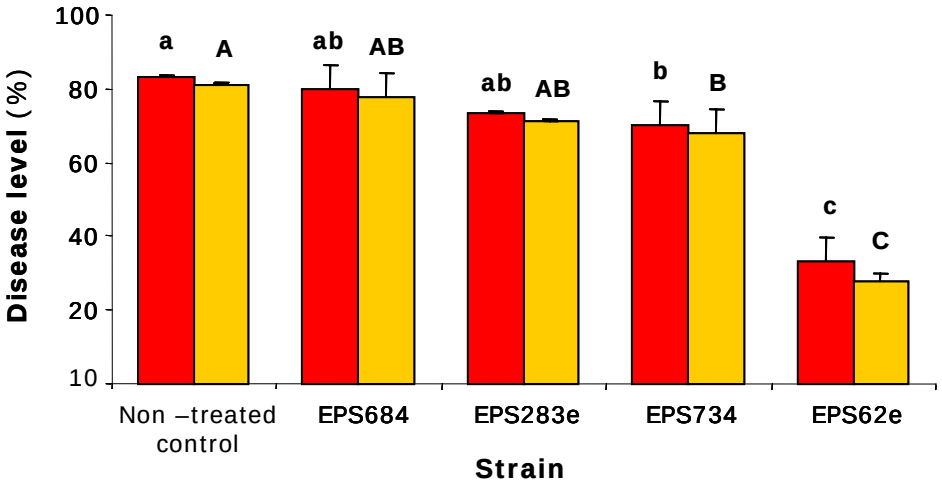

Figure 3.26. Effect of treatments with different antagonists on disease incidence (red series) and severity (yellow series) of infections in Doyenne du Comice pear flowers inoculated with *E. amylovora*. Data correspond to the second trial. Flowers were treated by spraying with antagonist the suspension at 10^8 cfu·mL^{-1} and inoculated 24 h later by deposition of 10 µl of a pathogen suspension at 10^7 cfu·mL^{-1}. Incidence and severity were assessed after 8 days at 21 ºC and high relative humidity. The same (capital and non capital) letter indicates that significant differences do not exist (P>0.05) according to Tukey's test.

4.2. Young shoot assays

In the first trial, treatments by spraying EPS62e at concentrations of 10^8 and 10^9 cfu·mL^{-1} showed a significant reduction of the incidence and severity of *E. amylovora* infections when the pathogen was inoculated in leaf wounds at 10^7 cfu·mL^{-1} (*Table 3.17*). No significant differences were observed between both concentrations of EPS62e used (10^8 and 10^9 cfu·mL^{-1}). The efficacy in reduction of incidence and severity obtained by the treatment at 10^9 cfu·mL^{-1} was 97% and 98%, respectively. While, when EPS62e was applied at 10^8 cfu·mL^{-1}, the efficacy obtained was 86% and 98%.

In the second trial, a treatment based on application of EPS62e at 10^8 cfu·mL^{-1} was assessed, and significant differences ($P<0.001$) with the non-treated control were observed for the severity and incidence (*Table 3.17*). In plants treated with strain EPS62e, symptoms of infection were not observed after 10 days of pathogen inoculation, thus obtaining a full control of the disease.

Finally, a third trial using 10^8 and 10^9 cfu·mL^{-1} was performed to verify the effect of concentration. Both treatments reduced significantly ($P<0.001$) the incidence and severity of infections in young shoots (*Table 3.17*). Besides, significant differences between the treatments were observed. The efficacy was around 96% when EPS62e was applied at 10^8 cfu·mL^{-1}, and around 70% when was applied at 10^7 cfu·mL^{-1}. In *Figure 3.27*, the differences between plants can be observed. From the 3 trials performed it is concluded that strain EPS62e gave consistent control of shoot blight. Besides, the minimum concentration which gave optimum control was 10^8 cfu·mL^{-1}.

Table 3.17. Effect of EPS62e concentration on the incidence and severity of shoot blight in several trials

Treatment	*First trial*[x]		*Second trial*		*Third trial*	
	Incidence[y] (%)	**Severity** (%)	**Incidence** (%)	**Severity** (%)	**Incidence** (%)	**Severity** (%)
Non-treated control	86 a	79 a	97 a	83 a	100 a	94 a
10^7 cfu·mL-1	-	-	-	-	38 b	27 b
10^8 cfu·mL-1	3 b	2 b	0 b	0 b	3 c	2 c
10^9 cfu·mL-1	1 b	1 b	-	-	-	-

[x]First trial was performed on detached pear branches (cv. Doyenne du Comice) with buds forced to shoot, while second and third trial were performed on whole pear plants (cv. Conference) in container.
[y]Wounded young shoots were treated by pulverization with EPS62e suspension at different concentrations and inoculated after 24 h by deposition of 10 µl *E. amylovora* EPS101 suspension at 10^7 cfu·mL^{-1} on the wounds produced in the leaves. Plants were incubated during 10 days at 21 ºC and high relative humidity. Means of the same column followed by different letters are significantly ($P\leq0.05$) different according to Tukey's test.

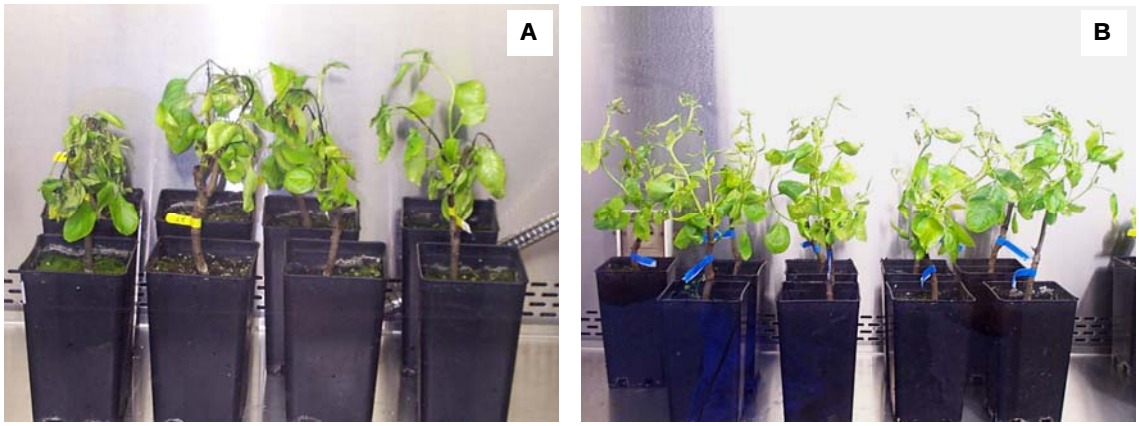

Figure 3.27. Non-treated pear plants (cv. Conference) (**A**) and treated with a suspension of strain EPS62e (**B**). Plants were treated by spraying of EPS62e at 10^8 cfu·mL^{-1} before the inoculation with *E. amylovora* EPS101 at 10^7 cfu·mL^{-1} after 24 h of preincubation. Plants were incubated during 10 days at 21 ºC, high relative humidity and photoperiod with 16 h of light.

DISCUSSION

1. Selection of potential biocontrol agents of fire blight

In studies dealing with development of biocontrol agents, the first step is the screening of microorganisms to select strains with ability to inhibit the growth of the pathogen or the disease it causes (Andrews, 1992; Bonaterra, 1997; Mercier and Lindow, 2001). The procedure used to collect isolates has an important influence in the final success. However, this aspect has been often considered secondary.

In the present work, we selected isolates from rosaceous plants mainly of *P. fluorescens* and *E. herbicola* species. These species were selected because most of biocontrol agents described to control fire blight corresponded to them (Beer *et al*., 1987; Wilson *et al*., 1992; Mitchell *et al*., 1996; Kearns and Mahanty, 1998). Besides, *P. fluorescens* and *E. herbicola* are frequent colonizers of the ambient where *E. amylovora* develops the infection process and produce a wide range of antibiotic compounds and antimetabolites (O'Sullivant and O'Gara, 1992; Wodzinski and Paulin, 1994; Bonaterra, 1997; Ellis *et al*., 2000; Badosa, 2001).

Once a collection of 533 isolates was constituted with isolates obtained during this work and with others from the research group collection, a method for screening the antagonists of *E. amylovora* had to be selected. One of the first screening assays described to select potential biocontrol agents was based on the production of a bacteriocin by *E. herbicola* against *E. amylovora* using the agar diffusion test (Beer *et al*., 1984b). However, it was rapidly noted that there was no correlation between the inhibition *in vitro* and the ability to inhibit the

pathogen in the field (Beer *et al*., 1984b). Besides, the agar diffusion test selects antagonists only for the capacity to inhibit the pathogen by means of antibiosis. Therefore, in using this screening method, many potential biocontrol agents that do not develop antibiosis may be discarded (Mercier and Lindow, 1996). In addition, this selection process does not take into account the interaction of the antagonist with the host nor some characteristics that must possess the biocontrol agent, like the colonization ability to succeed in the biocontrol of pathogen on the plant host. However, the antibiosis plays an important role in some interactions and its involvement in the inhibition of the pathogen should be tested on representative media (Andrews, 1992). In contrast, the screening of *E. amylovora* antagonists using the immature pear fruits assay (Beer and Rundle, 1983) is useful because it allows selecting the antagonists taking into account the interaction host cell-pathogen-antagonist. Almost all the biocontrol mechanisms involved in the biocontrol of *E. amylovora* are favored in this system, like the competition for nutrients, space exclusion, antibiosis, and induction of defense responses in the host. For these reasons, the first screening developed in the present study was based on the immature pear fruit assay. However, several researchers have observed that no correlation is found between the ability of different strains of *E. herbicola* to inhibit *E. amylovora* in fruits and their ability to inhibit *E. amylovora* in flowers (McLaughlin and Roberts, 1992; Wilson *et al*., 1990; Pusey, 1996).

Every laboratory which used the immature pear test modified it slightly to meet its requirements. In our laboratory, entire fruits are used instead of slices of pear used by most of laboratories (Ishimaru *et al*., 1988; Wilson *et al*., 1990; Vanneste, 1996). In the present work wounded fruits were treated by immersion in the potential antagonist suspension at 10^8 cfu·mL^{-1}, and *E. amylovora* was inoculated in each wound by deposition of one drop of 10 µL of the pathogen suspension at 10^7 cfu·mL^{-1} in accordance with the results obtained for the optimization of blossom and shoots model pathosystem performed in Chapter 2 and according to methods reported by other researchers (Rundle and Beer, 1987; Wilson *et al*., 1992; Pusey, 1997).

Another important aspect in the assay procedure is the timing between the application of the antagonists and the pathogen inoculation (before, simultaneously, and after) because affects disease control levels. The pre-emptive application of the antagonist is the strategy to be used in the fire blight control because it is expected that antagonists lose their efficacy when coinoculated or postinoculated with the pathogen (Wilson and Lindow, 1993; Lindow *et al*., 1996). Moreover, the best moment of the antagonist application can differ according to the control mechanism used by the antagonist. Thus, if the control mechanisms developed are the competition or induction of defense responses in the host (e.g. *P. fluorescens* A506) disease control is not performed when the antagonists are coinoculated with the pathogen (Tharaud *et al*., 1997; Stockwell *et al*., 1999). In the present study, isolates were treated 24 h prior to the pathogen inoculation to favor wound colonization by the putative biocontrol agents previous to the establishment of the pathogen and to avoid discarding potential antagonists, those who use as mechanism competition or induction of defense responses in the host.

Using the modification of whole immature pear fruit assay with preinoculation, most of the 533 isolates evaluated reduced the incidence of *E. amylovora* infections in immature pear fruits, but only a 10% presented a high efficacy. This indicates that it is relatively easy to find antagonists effective against *E. amylovora* in immature pear fruits but it is more difficult to find antagonists developing high levels of disease control. For this reason, the development and the improvement of screening methods to select better antagonists is an important challenge (Lindow *et al*., 1996). Finally, 61 antagonists were selected from the initial 533, presenting high efficacy in the immature pear fruit assay. These antagonists were used for further studies in order to select a potential biocontrol agent of fire blight and to improve the knowledge on the characteristics involved in the biocontrol and the performance of screening methods.

The immature pear fruit assay have an inherent variability because there are many uncontrolled factors mainly due to the three living organisms used (the host, the pathogen and antagonist). Thus, the inhibition of *E. amylovora* in many cases is not the product of the intrinsic characteristics of the antagonist, but of the uncontrolled factors that favor the antagonists or prejudice the pathogen. For this reason, two consecutive trials were done to test the consistency of the effect of the most effective strains.

The second trial was developed using the same procedure as for in the first trial but changing the pathogen strain. Thus *E. amylovora* EPS101 was used instead of CUCM273 in the second trial. Strain EPS101 was selected because it was very different to strain CUCM273. CUCM273 is serologically different to most of *E. amylovora* strains due to it is not detectable by DASI-ELISA targeted to the typical *E. amylovora* strains, and it does not present the typical white, domed, shiny, mucoid colonies (levan type) in sucrose nutrient agar due to it does not produce the amylovoran; EPS101 presents the common characteristics of most of *E. amylovora* strains. In the second trial, only 10 out of the 61 strains retested against EPS101 maintained a high efficacy in the inhibition of *E. amylovora* infections in immature pear fruits. Therefore after the second trial 10 out of 533 were retained, corresponding to 1.5% efficiency in the screening process. This efficiency is in accordance with studies performed on other pathosystems using *ex vivo* assays such as against *Stemphylium vesicarium* in pear leaves (Montesinos *et al*., 1996) or against *Sclerotinia sclerotiorum* in bean buds (Yuen *et al*., 1994) with efficacies of 7% and 3%, respectively.

The efficacy of the antagonists in immature pear fruits decreased from a maximum of 100% in the first trial to a 65% in the second. Differences can be attributed to a decrease in the host tissue sensibility or the pathogen strain. Thus, the fruits were gathered in different year, and the pathogen strain was changed and consequently its virulence and its sensibility to the antagonists. Severity and incidence of fire blight depend on the state of maturation of plant tissue, obtaining greater levels of disease with younger tissues. Nevertheless, differences in the virulence of both strains has been observed in the previous Chapter, being strain EPS101 more virulent than CUCM273 according the virulence parameters estimated from disease progression curves.

Even with the above mentioned reduction in the efficacy levels, four *E. herbicola* strains (EPS156e, EPS237e, EPS283e, EPS438) and six *P. fluorescens* (EPS62e, EPS82e, EPS372, EPS538, EPS684 and EPS734) significantly reduced the incidence of *E. amylovora* EPS101 infections in immature pear fruits. These strains were selected as putative biocontrol agents because they developed a highly consistent efficacy (around 60-100%) in immature pear fruits. Nevertheless, strains EPS237e and EPS372 were discarded to present undesired characteristics because EPS237 is ice nucleation active and EPS372 induce HR in tobacco.

The model of immature pear fruit is far from the natural conditions and some studies indicate that no correlation exist between the ability of different isolates to inhibit *E. amylovora* in fruits and their ability to inhibit *E. amylovora* in flowers (Wilson *et al.*, 1990; Pusey, 1996). Therefore, before considering these antagonists as serious biocontrol agents of fire blight, it is necessary to evaluate their aptitudes in more representative models such as flowers or shoots.

The flowers model was selected because some studies indicate that fire blight is controlled when antagonistic bacteria are applied to and become established on the stigmatic surfaces of pear and apple blossoms prior to colonization by *E. amylovora* (Beer *et al.*, 1984; Wilson and Lindow, 1993; Lindow *et al.*, 1996). Moreover, these studies have found a correlation between the ability of antagonists to colonize the flower surfaces and the capacity to suppress populations of *E. amylovora*. In the present study, the blossom assay was used to confirm the potential of eight strains (EPS156e, EPS283e, EPS438, EPS460, EPS62e, EPS538, EPS684 and EPS734) as biocontrol agents of *E. amylovora*. The assay was performed according to the conditions determined in Chapter 2. The levels of disease control observed on flowers were lower than on immature pear fruits indicating that flowers are more susceptible to *E. amylovora* infections. However, in the first trial the strains EPS283e, EPS684, EPS734 and EPS62e significantly inhibited *E. amylovora* with efficacies ranging from 34 to 78%, while, in the second trial, only strain EPS62e significantly inhibited *E. amylovora* with an efficacy of 69%. EPS62e was the most effective strain and the only that exhibited consistent high efficacy in pear blossoms. In contrast, strains EPS460, EPS438 and EPS156e were unable to control blossom blight, probably due to their lack of capacity to colonize flowers or to inhibit *E. amylovora* into the flowers surface. Strain EPS538 was discarded because it produced a slight necrosis on flowers.

The assay performed in young shoots has been proposed in the present study as an advance on the selection of biocontrol agents of fire blight. It was performed to evaluate the capacity of strain EPS62e to colonize efficiently leaves and to exclude *E. amylovora* from leaf wounds. Disease incidence and severity in young shoots assay depends on the phenological stage of the plants. Thus, using youngest shoots, disease levels reached around 100% of incidence while were reduced in older the shoots. Consequently, young shoots at the same developmental stage were used in this assay. The homogeneity of shoots was thoroughly selected to avoid variability due to plant material. The levels of disease control on shoots were higher than on immature pear fruits and pear flowers. This is attributed to the greater difficulty of *E. amylovora* to colonize plant surface than flowers or immature fruit wounds,

probably due to a greater limitation on nutrients. Besides, this also indicates that shoots are less sensible to fire blight than flowers or immature fruits. Three trials were performed for evaluation of the efficacy of EPS62e at three inoculum concentrations. EPS62e controlled efficiently infections caused by *E. amylovora* EPS101 in all the trials, with an efficacy around 90% when it was applied at 10^8 and 10^9 cfu·mL^{-1} and around 70% at 10^7 cfu·mL^{-1}. Therefore, it is concluded that strain EPS62e control shoot blight with high efficacy and consistency and optimum control levels at 10^8 cfu·mL^{-1}.

The results observed in the efficacy assays indicate that a prior colonization of the wounds by EPS62e resulted in the inhibition of *E. amylovora* infections in young shoots. This inhibition can be due to pre-emptive utilization of nutritional resources required for the pathogen or to interference with entry sites necessary for the pathogen infection and disease development or antibiosis against *E. amylovora*. This has been described for *P. fluorescens* A506 in pear blossoms (Stockwell *et al*., 1999). Thus, the study of the mechanism of control developed by EPS62e is necessary to provide information which may enhance the efficacy of this strain or helping the selection of strains more effective or complementary to EPS62e.

2. Search of specific traits associated to biocontrol of fire blight

Although, there have been a considerable development in biological control, and several biocontrol agents are now available commercially most of them in USA (Fravel *et al*., 1999), there has been little progress made on screening methods by which potential antagonists can be identified (Blakeman and Fokkema, 1982; Andrews, 1992). The finding of markers or patterns of traits associated to a biocontrol agent could be useful to optimize screening methods. In the present study, the sixty one most effective strains were characterized using the API system, *in vitro* antagonism on agar media against different plant pathogens, production of interesting compounds (antibiotics, siderophores, chitinases, indolacetic acid), level of inhibition of infections in immature pear fruits, hypersensitivity reaction and ice nucleation activity in order to find specific traits associated to biocontrol.

The study showed that strains of the same species were very similar and although some groups were discriminated, a relation with the high efficacy in the inhibition of infections was not found.

A direct relation between the activity *in vitro* against *E. amylovora* and the inhibition of *E. amylovora* in immature pear fruits was not found. Only 23% of selected strains, accounting for *P. fluorescens* and *E. herbicola* species, presented *in vitro* activity against *E. amylovora*, and most of the antagonists with high and consistent efficacy in immature pear fruits did not develop *in vitro* antagonism against *E. amylovora*. These results are in accordance with other studies suggesting that antibiotic production is important in the biological control of *E. amylovora*, but additional mechanisms can be involved (Vanneste *et al*., 1992; Wilson *et al*., 1992; Nuclo *et al*., 1998). In the present study, competition for nutrients and space or induction of defense responses in the host could be the main mechanisms involved in the

inhibition of *E. amylovora* infections in immature pear fruits (Wilson and Lindow, 1993; Tharaud *et al*., 1997). Our results also indicate that the immature pear fruit assay permits the identification of antagonists of *E. amylovora* independently of the mode of action developed, in contrast with the opinion of some researchers that immature pear fruit assays are useful for conducting a preliminary screen of the efficacy of antagonist strains for which antibiotic is considered the principal mode of action (Vanneste, 1996).

Although antibiotic-producing strains have been shown to control fire blight (Beer *et al*., 1984; Wilson *et al*., 1992), some of the strains selected in our study as efficient biocontrol agetns did not produce antibiotics and would have probably been missed if the screening process used has been based on antibiosis. These results are in accordance with the obtained by Mercier and Lindow (2001), where none of strains selected exhibiting high efficacy in the reduction of blossom blight produced antibiotics neither antagonism on agar media.

It is important also to notice that most of antagonists inhibiting *E. amylovora* in agar medium, lost this ability upon iron amendment to the medium. Therefore this inhibition can be attributed to siderophore production. In contrast, when the inhibition of *E. amylovora* was not suppressed by iron, antibiotics production is expected. In accordance with this result, other authors have described several strains of *E. herbicola* that synthesize compounds with inhibitory activity against *E. amylovora* like herbicolacin 112Y (Wodszinski *et al*., 1987), herbicolin O and I (Ishimaru *et al*., 1988), a β-lactam antibiotic (Kearns and Mahanty, 1998), and pantocin A and B (Wright *et al*., 2001).

In relation to the production of interesting compounds, all the strains except EPS10e produced siderophores while chitinases were only produced by *S. marcescens* strains. Besides, only two strains of *P. fluorescens* produced cyanide, six produce indolacetic acid, and none of the antagonists produced the antibiotics 2,4-diacetylphloroglucinol, phenazine-1-carboxylic or pyrrolnitrin. No relation was found between the production of these compounds with the inhibition of infections in immature pear fruits.

A correspondence analysis using the traits of the antagonists including the efficacy in the *E. amylovora* inhibition in immature pear fruits have shown that the ability to inhibit infections at high and medium efficacy levels was related to the species *P. fluorescens* and *E. herbicola* and to isolates obtained from rosaceous plants. This is in accordance with the previous results obtained by Bonaterra (1997) in which the low efficacy was related to *S. marcescens,* to isolates from non rosaceous plants, antagonism on agar media against *P. expansum,* and chitinase production. Similar results were obtained by Badosa (2002) that compared phenotypic and genotypic characteristics of a selected group of fluorescent *Pseudomonas* by means of a polyphasic approach in order to establish relationships with the ability of being biocontrol agent of plant growth promoting bacteria. Also Mavrodi *et al*. (2001) did not observed a relationship between production of phloroglucinol by *P. fluorescens* strains with biocontrol of several soilborne fungal pathogens. In contrast, other studies reported specific markers associated to biocontrol agent ability. This is the case of Ellis *et al*. (2000) that reported accumulation of C17:0 cyclopropane fatty acid and production of hydrogen cyanide

by fluorescent pseudomonads correlated significantly with biological control activity and with antagonism against *Pythium ultimum*.

In conclusion, no relationship was observed between the high efficacy in the inhibition of infections caused by *E. amylovora* in immature pear fruits and the API system profile, *in vitro* antagonism on agar media against different plant pathogens and production of antimetabolite compounds (antibiotics, siderophores, chitinases, indolacetic acid). Therefore, a pattern of common characteristics among the antagonists with high efficacy against *E. amylovora* on immature pear fruits was not found.

CHAPTER 4

Characterization and putative mechanisms of action of *P. fluorescens* EPS62e

INTRODUCTION

For the commercial development of a biocontrol agent, it is necessary to more fully understand its ecology and interactions with the pathogen, host plant, surrounding plant surface microbial communities and environment. Ideally, the antagonist must survive and control the pathogen within the particular conditions of their ecosystem. Moreover, the antagonist must be at adequate population levels and be capable of effectively interacting with the pathogen or host plant to provide acceptable disease control. Finally, patenting and registration procedures for commercial delivery requires the set up of specific methods of strain identification and traceability studies that permit to distinguish introduced strains from indigenous (Cook and Baker, 1983; European Commission, 2002; Montesinos, 2003).

Once a putative biocontrol agent has been selected, it is necessary to identify and to characterize the putative biocontrol agent to discard deleterious or pathogenic species, to improve its biocontrol activity and to determine the mode of action that will affect the application strategy. One of the characteristics that must possess a good biocontrol agent is a broad range of activity against pathogens (Powell, 1993). Another important characteristic is the compatibility with chemical products frequently used in crop protection. This is necessary to become compatible the biocontrol with the standard orchard management and to apply integrated control strategies.

Understanding the mechanism of action involved in biocontrol processes can permit the establishment of optimum conditions for the interaction between the pathogen and the biocontrol agent and is important for implementing biocontrol in a given pathosystem (Bonaterra *et al*., 2003). Besides, knowledge of mechanism of action can provide much insight into where and when the interaction occurs and how the pathogen will be affected.

There are several mechanisms involved in biological control such as the inhibition of the pathogen by antibiotic compounds, direct interaction, induction of plant resistance mechanisms, and competition for space and limited resources. However, it is generally assumed that the mode of action of many biocontrol agents involves complex processes in which one or more mechanisms play role in disease control (Whips, 2001).

It is difficult to determine the mechanism of a biocontrol agent, because in most cases several mechanisms implicated can be correlated (Fravel, 1988) and may be conditioned to environmental conditions (Weller, 1988). Whatever alteration of these conditions can interfere in the mode of action of a biocontrol agent, and can vary the role of each mechanism in disease control (Epton *et al*., 1994). Thus, to determine the contribution of possible mechanisms, it is necessary to separately assess the role of each individual mechanism (Wright and Beer, 1996).

In addition, it is difficult to determine the mechanism of action under field conditions, and for this reason *in vitro* assays or assays performed under controlled conditions are used. Although, these assays may not reflect which happens under field conditions, and the interpretation of results obtained from these studies often raises unanswered questions (Chalutz and Droby, 1998). For this reason, in most cases the mechanism of biocontrol is not fully explained.

The development of new molecular biology techniques allows the confirmation of a given mechanism in the inhibition of a pathogen. Thus, the role of antibiosis can be demonstrated by the evaluation of the efficacy of mutants unable to produce the antibiotic in comparison to the wild type (Lam and Gaffney, 1993). Several studies based on deficient mutants in antibiotic production, obtained by means of transposon mutagenesis (Tn5), have allowed demonstrating the implication of antibiosis in the biocontrol. For example, production of PCA has been implicated in the biocontrol of *Gaeumannomyces graminis* by *P. fluorescens* 2-79 (Tomashow and Weller, 1988). Also the biocontrol developed by *P. fluorescens* F113 on the soft rot potato pathogen *E. carotovora* subsp. *atroseptica* has been attributed to production of the antibiotic Phl (Cronin *et al.*, 1997). Although, in some cases the implication of antibiosis does not explain biocontrol. This is the case of the pyoluteorin and pyoverdine *P. fluorescens* deficient mutant (strain Pf-5) that loses the capacity to inhibit *Pythium in vitro* but maintains the capacity to suppress the disease indicating that there are other mechanisms involved in the biocontrol instead of antibiotic production (Kraus and Loper, 1992).

Nowadays, it is also possible to determine the implication of the induction of plant resistance in biocontrol, because many pathway messengers and PR proteins, such as plant produced chitinases (PR-3, 4, 8 and 11), β-1,3-glucanases (PR-2), proteinase inhibitors (PR-6), or peroxidases (PR-9) (van Loon, 1997) are known. There are techniques to determine the induction of SAR and ISR by means of detection of accumulation of PR proteins or pathway

messengers or by quantification of the specific expression of pathogenesis-related mRNAs (Schweizer *et al.*, 1998; Redmand *et al.*, 1999; van Loon and van Strien, 1999).

More difficult is to demonstrate that competition is responsible of biocontrol. Janisiewicz *et al*. (2000) have developed a simple approach with an *in vitro* system closely resembling *in vivo* conditions to study competition for nutrients as the mechanism of biological control of fruit decays. Other studies applied this approach to determine the implication of antibiosis and direct interaction in biocontrol of postharvest diseases (Bonaterra *et al*., 2003). Even using new approaches, the determination of the involvement of these mechanisms in disease reduction is difficult when the antagonist develops an additional biocontrol mechanism, like antibiosis. In this case, it is very difficult to untie the mechanisms involved because probably they have a synergic effect.

Several mechanisms have been proposed to explain the inhibition of *E. amylovora* by different strains of biological control agents. However, the only mechanisms proven in biological control of fire blight are the competition for nutrients and space, and production of an antibiotic type molecule (Vanneste, 1996). The implication of antibiosis in the inhibition of *E. amylovora* has been reported in many *E. herbicola*, such as strain Eh112Y (Wodzinski *et al.,* 1987), C9-1 (Ishimaru *et al.* 1988), Eh318 (Wodzinski *et al.*, 1989), HL9N13 (Wilson *et al.,* 1992), Eh252 (Vanneste *et al.,* 1992) and Eh1087 (Kearns and Hale, 1996). In contrast, *P. fluorescens* A506 is the only biological control agent which does not exhibit antibiosis to *E. amylovora* when tested on different media, including minimal medium (Lindow, 1985). In this case, the depletion of some factors necessary to the pathogen growth seems the cause of the reduction fire blight incidence (Wilson and Lindow, 1993).

Biological control levels depend greatly on pathogen and biocontrol agent concentration (Baker, 1978; Adams, 1990; Baker, 1990; Cartwright and Benson, 1994; Raaijmarkers et al., 1995). For this reason, the development of a biocontrol agent requires accurate data on optimal effective dose and a complete characterization of dose-response relationships with disease control. Knowledge of antagonist-pathogen inoculum density relationship permit to determine the population levels of the antagonist that are required to achieve adequate disease control, as well as the pathogen inoculation levels at which the antagonists will or will not be effective (Montesinos and Bonaterra, 1996; Larkin and Fravel, 1999).

Many studies determine efficacy at increasing biocontrol agent concentrations for a single concentration of the pathogen (Hong *and* Michailides*,* 1998; Zhou *et al.,* 2001). However, this procedure is not sufficiently accurate because the efficacy of biological control agents is dependent on both, the pathogen and the biocontrol agent concentrations (Johnson, 1994; Montesinos and Bonaterra, 1996). There is a need for objective means to fit data on infectivity titration of pathogens and biocontrol agents to suitable models that may provide dose-response surfaces and parameters describing the virulence of the pathogen and the efficiency of the biocontrol agent (Montesinos and Bonaterra, 1996). In these models, the degree of disease control obtained with a biocontrol agent depends on the density of biocontrol agent, the density of pathogen, the efficiency of biocontrol agent in suppressing

the pathogen, and the proportion of the pathogen population that is potentially affected by the agent.

Models based on hyperbolic saturation of the host by the pathogen (Fukui *et al*., 1994) and on the lognormal probability distribution (Rouse *et al.*, 1985) were used to relate the dose of pathogen to disease levels. These models have been adapted to biocontrol studies and are interesting because they provide valuable parameters, such as the median effective dose (ED_{50}) and the relative efficiency (Montesinos and Bonaterra, 1996). Such models are useful for comparing different biocontrol agents and systems, help in understanding the factors affecting biocontrol interactions, and are useful in predicting biocontrol efficacy under varying conditions.

Detection and traceability of biocontrol agents are essential for colonization studies and for evaluating their impact in the environment. Several studies of biocontrol used a viable count method based on semi-selective culture media or even colony appearance, for detection and quantification of introduced strains (Janisiewicz and Marchi, 1992; Benbow and Sugar, 1999; Nunes *et al.*, 2002). However, this method may have interference from the autochthonous microbiota corresponding to the same species of the strain used as biological control agent. To avoid this problem many field studies dealing with monitoring introduced biocontrol agents or pathogens used spontaneous mutants harboring a selectable antibiotic resistant marker in combination with a selective medium amended with the antibiotic (Cody *et* al., 1987; Wilson and Lindow, 1993; Johson, 1994; Montesinos *et al.,* 1996).

Nowadays, novel molecular biology-based techniques such as DNA probing and marker gene tagging have recently been developed as specific methods to identify and quantify populations of specific microorganisms in the environment (Jansson and Prosser, 1997). One of the most promising markers is the *gfp* gene, encoding the green fluorescent protein (GFP). An advance of GFP is that, unlike other biomarkers, it does not require any substrate or additional cofactors in order to fluoresce (Chalfie *et al*., 1994). In addition, GFP-fluorescent bacterial cell populations can be analyzed and quantified by means of flow cytometry (Tombolini *et al.*, 1997). Nevertheless, the use of this kind of biomarkers involves the genetic modification of the bacterial strain, and therefore a genetic modified organism (GMO) is obtained with all its implications such as difficulties for further practical applications and demonstrates the fitness of the modified strain.

Other type of genetic markers are based on the specific identification methods based on DNA sequences. DNA markers minimize the difficulty of typeability and lack of reproducibility common in the use of phenotypic-based methods (Olive and Bean, 1999). A DNA marker can be obtained by gene insertion (Chand-Goyal *et al.*, 1999), but this type of DNA marker involves genetic modification of the organism, which would introduce difficulties for further practical applications. Moreover, the application of this method also requires that wild type and engineered strains have the same fitness properties *in situ*, which is still difficult to demonstrate. In contrast, identification of a DNA-based typing on natural polymorphisms overcomes these problems. One of the examples is the use of non-quantitative methods

based on RFLP patterns using Pulsed Field Gel Electrophoresis to confirm the identity of the biocontrol agent introduced. These methods can be used to monitoring introduced biocontrol agents when are combined with the use spontaneous mutants harboring a selectableantibiotic resistant marker in combination with a selective medium amended with the antibiotic (Wilson and Lindow, 1993; Montesinos *et al*., 1996). More recently, the use of Sequence Characterized Amplification Region (SCAR) markers allow a reproducible amplification of one single fragment increasing the specificity and sensibility of the biocontrol agent detection. Although, this technique makes impossible to differentiate the viable cells from death cells which makes necessary the development of a monitoring method combining dilution plating on a semi-selective medium and strain-specific identification of colony forming units with the SCAR marker (de Clercq *et al*., 2003).

In the previous chapters of this PhD, EPS62e was selected over 533 antagonists as a potential biocontrol agent against fire blight after showing high efficacy and consistency on the inhibition of *E. amylovora* infections on immature pear fruits, pear flowers and young pear shoots. Therefore, this strain should be characterized in detail, and the action mechanisms preliminary characterized.

OBJECTIVES

This chapter aim to increase the knowledge on several characteristics of the strain EPS62e important for a future development as a biological control of fire blight.

The objectives of this section were to:

- Identify and characterize strain EPS62e using the electrophoretic pattern of macrofragments of digestion of genomic DNA, its range of action against strains of *E. amylovora* and other plant pathogens, and its sensitivity to several chemical commercial pesticides.

- Study the influence of the biocontrol agent and pathogen concentrations on fire blight control.

- Determine the putative mechanisms of action involved in the biocontrol of *E. amylovora*.

- Study colonization and survival of strain EPS62e in different plant organs under controlled environment and field conditions.

MATERIALS AND METHODS

1. Identification and characterization of *P. fluorescens* EPS62e

1.1. General description

P. fluorescens EPS62e is a Gram negative motile rod, without ice nucleation activity, and does not induce hypersensitive reaction on tobacco and geranium. Moreover, it does not have the ability to produce the common antimicrobial compounds described on *P. fluorescens*. EPS62e was isolated from a pear fruit from which the extract was effective in inhibition of *E. amylovora* infections in immature pear fruits (cv. Passe Crassane). This strain has shown a consistent efficacy in inhibition of infections caused by different *E. amylovora* strains in immature pear fruits, pear flowers and young shoots. For this reason it has been selected as a potential biocontrol agent of fire blight (see *Chapter 3*). A spontaneous mutant resistant to 50 $\mu g \cdot mL^{-1}$ of nalidixic acid which retains phenotypical characteristics and performance of the parental strain, was used in the present study. The mutant was obtained by selection of a spontaneous resistant colony obtained by seeding a confluent culture in LB agar supplemented with nalidixic acid using the gradient method. Cultures were grown in LB agar amended with nalidixic acid and stored in 20% glycerol at -80 ºC.

1.2. Strain Identification

P. fluorescens EPS62e was identified by means of API 20E and 20NE systems and fatty acids profile.

1.2.1. API 20E and API 20NE systems

The methodology used was the same described in *section 3.1* from *Chapter 3.*

1.2.2. Fatty acids profile

Extraction of fatty acids was performed as described by Miller and Berger (1985) with minor modifications. A large loopful (one plate quadrant) of EPS62e grown for 24 h at 28 ºC in Trypticase Soy Broth (TSBA) agar (*Annex 2*) was placed into a tube containing 1 mL of 15% NaOH on 50% methanol. Fatty acids were saponified by heating the tube for 30 min in a 100ºC waterbath, with two brief periods of mixing during heating. Following cooling, 2 mL of 6 N HCl in 50% methanol were added. The tubes were heated at 80ºC for 10 min to form methyl esters of the free fatty acids. Extractions into 1 mL of hexane/methyl tert-butyl ether (MTBE) (1:1) were followed by discarding of the aqueous phase and washing of the organic extract with 3 mL of 1.2% aqueous NaOH (Sasser, 1990). The fatty acids in the hexan/ether extract were then assayed with a gas chromatograph (HP-5890 series II, Hewlett Packard, USA). Fatty acid pattern was introduced in control software (HP Gas Cromatography-Chemical Station Rev. A.07.01 (682) 1990-1999, Hewlett Packard, USA), and then processed by Sherlock software (Microbial Identification System, MIDI, Inc., 125 Sandy Drive, Netwark,

USA). Gas chromatography was performed in the laboratory of the "Unitat del Servei de protecció dels Vegetals" of the "Departament d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya" (DARP).

1.3. Characterization

EPS62e was characterized by DNA fingerprinting, tolerance to chemical pesticides and spectrum of antagonistic activity against *E. amylovora* strains and other bacterial plant pathogens.

1.3.1. Molecular fingerprinting using MRFLP-PFGE

The electrophoretic pattern of macrofragments of digestion of genomic DNA with the restriction endonuclease *Swa* I was determined by means of the procedure described in *section 3.3* from *Chapter 2***.** Pure cultures grown in LB broth were centrifuged, included in agarose blocks, and then lysed in digestion buffer (*Annex 1*) for 48 h at 56 ºC. Embedded DNA was digested with *Swa* I at 25 ºC for 24 h. Resulting fragments of DNA digestion were transferred to an agarose gel and separated by means of CHEF procedure of electrophoresis in a pulsating field using a constant pulse of 25s for 21 h at 200 V and 120 mA. The PFGE pattern of EPS62e was compared to the patterns obtained for several strains of *P. fluorescens* corresponding to biocontrol agents provided by other researchers (BL915, CHA0), type strains (CECT844, CECT378, CFBP2128, CFBP2131) or natural isolates obtained during this study (EPS372, EPS818) (see *section 2* from *Chapter 3*).

1.3.2. Evaluation of sensitivity to several chemical pesticides

The tolerance of EPS62e to several chemicals used in plant protection was evaluated by means of the agar incorporation test. Six products were used at the recommended field doses described by the manufacturers (*Table 4.1*). Solutions were prepared at the corresponding dose in 2 mL of sterile distilled water and incorporated in 18 mL of melted CYE agar (*Annex 2*) and dispensed in Petri dishes. EPS62e colonies were transferred to the surface of CYE plates with toothpicks, and incubated at 25 ºC. Growth inhibition was assessed after 48 h. Triplicates for each chemical were performed.

Table 4.1. Chemical pesticides tested

Active ingredient	**Product**	**Company manufacturer**	**Type of product**	**Recommended dose** (g a.i.·L^{-1})
Kasugamycin	Kasumin	Lainco S.A.	Antibiotic	0.5
Streptomycin	Streptomycin	Sigma	Antibiotic	0.5
Copper sulphate	Bordeaux mixture	Industrias Químicas del Vallés, S.A. (IQV)	Copper compound	3.0
Copper oxychloride	Cuproflow	Caffaro S.A.	Copper compound	3.5
Copper hydroxide	Hidrocobre	IQV	Copper compound	3.0
Fosetyl-aluminium	Alliette	Aventis S.A.	Phosphonate	3.0

1.3.3. Spectrum of antagonistic activity on agar media

In a first trial, antagonistic activity of EPS62e on agar media was determined against 16 strains of *E. amylovora* isolated from different host plants and geographical locations. These strains had particular PFGE and API patterns, and different virulence degree (*Table 4.2*). In a second trial, antagonistic activity was also tested against 8 phytopathogenic bacteria including *Xanthomonas fragariae* (CFBP3549-95), *X. campestris pv. vesicatoria* (CFBP3275), *X. arboricola, P. syringae, Pseudomonas corrugata* (CECT124T), *Ralstonia solanacearum* (CECT125), *Agrobacterium tumefaciens* (CECT472), and *E. amylovora*. Besides, three pathovars of *P. syringae* were assessed, *phaseolicola* (CFBP3635-95), *tomato* and *syringae* (EPS94), as well as three strains of *E. amylovora*, EPS101, EPS100, and CUCM273.

Table 4.2. Characteristic traits of *E. amylovora* strains used in the study of the spectrum of antagonism of EPS62e against *E. amylovora*

Strain	Host plant of isolation	Country	PFGE pattern[x]	API pattern[y]	Virulence[z]
EPS100	*Pyrus malus*	Spain	4*	4	low
EPS101	*P. communis*	Spain	4*	1	high
CUCM273	*Malus sylvestris*	USA	-	2	high
CFBP1430	*Crataegus* sp.	France	3*	2	high
PMV6076	*Crataegus* sp.	France	3*	1	low
UPN529	*Pyracantha* sp.	Spain	4**	2	moderate
UPN611	*Cotoneaster*	Spain	4**	2	high
USV1000	*P. communis*	Spain	4*	2	moderate
OMP-BO1185	*Crataegus* sp.	Italy	3**	2	moderate
IVIA1614.2	*Crataegus* sp.	Spain	3**	2	moderate
Ea115.2	*Cydonia oblonga*	Bulgary	5**	1	low
EAZ4	*P. communis*	Spain	4**	1	low
NCPPB595	*P. communis*	England	1**	3	low
NCPPB1819	*Crataegus* sp.	USA	-	1	low
NCPPB2080	*P. communis*	New Zealand	2**	2	moderate
NCPPB3159	*M. sylvestris*	The Netherlands	1*	3	moderate

[x]PFGE pattern obtained during this study (*) or described by Jock *et al*., 2002 (**).
[y]API pattern determined during this study according to API 20E system (see *Chapter 2*).
[z]virulence degree based on the virulence parameters obtained from disease progression curves during this study (see *Chapter 2*).

Antagonism on agar was performed as described in *section 3* from *Chapter 3*. The antagonistic activity against *E. amylovora* strains was assessed on LB, GA, GA amended with iron (50 μM $FeCl_3$) (GA-fe), KB and KB amended with iron (50 μM $FeCl_3$) (KB-fe). The antagonistic activity against other plant pathogens was assessed on LB, Müeller-Hinton (MH), GA, GA-fe, KB and KB-fe. The diameter of the halus of inhibition was assessed after 48 h of incubation at 21 ºC. Three repetitions for each strain were performed.

1.3.4. Spectrum of action of inhibition of infection of immature pear fruits against a collection of strains of *E. amylovora*

The spectrum of action of EPS62e against 9 strains of *E. amylovora* (EPS101, EPS100, CUCM273, UPN611, USV1000, OMP-BO1185, Ea115.22, EAZ4, NCPPB2080) (*Table 4.2*) was evaluated on cv. Passe Crassane using the immature pear fruit assay described in *section 2.4* from *Chapter 2*. EPS62e was treated by deposition of 10 µL in each wound, and was applied at two concentrations, 10^7 or 10^8 cfu·mL^{-1}, while *E. amylovora* strains were inoculated with 10 µL of a suspension at 10^7 cfu·mL^{-1} 24 h after EPS62e application. Non-treated controls only inoculated with each pathogen strain were included. Experimental design consisted of three repetitions of three fruits with four wounds per fruit, per repetition, for each treatment.

Incidence of infections was calculated as infected wounds/total wounds inoculated and was assessed after 5 and 8 days of the pathogen inoculation. ANOVA was performed to test the effect of *E. amylovora* strain on the incidence of infections upon treatment with EPS62e. Means were separated according to the Tukey's test at $P \leq 0.05$.

2. Effect of pathogen and EPS62e cell concentrations on disease control

Dose-response experiments were used to determine the optimal effective dose of EPS62e to control EPS101 in immature pear fruits and in pear flowers. Besides, we determined the effect of the pear cultivar type in the inhibition of *E. amylovora* infections by EPS62e.

2.1. Pathogen strain

For this experiment *E. amylovora* EPS101 was used. EPS101 was isolated during the present work from a symptomatic Conference pear branch from a fire blight infected orchard in Lleida. EPS101 has been widely characterized in *Chapter 2* and is interesting because it presents high virulence according to the Composite Virulence Index described on immature pear fruits (see *Chapter 2*). Cultures were grown in LB agar and were stored until use in 20% glycerol at -80 ºC. Bacterial suspensions were prepared from cultures grown on LB agar plates at 25 ºC for 24 h. Cultures were scraped from the agar surface and suspended in sterile distilled water and the concentration adjusted to suitable concentrations.

2.2. Dose-response experiments on immature pear fruits

Incidence of infections in immature pear fruits was assessed at several concentrations of EPS101 (10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 cfu·mL^{-1}) and EPS62e (10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 cfu·mL^{-1}). Dose-response assays were done on four cultivars: Blanquilla, Conference, Doyenne du Comice and Passe Crassane. Fruits were processed as described in *section 2* from *Chapter 3* (*Figure 4.1*), but the application of EPS62e was performed by depositing 10 µL of the antagonistic suspension in each wound instead of the immersion of fruits in the antagonistic suspension. The experimental design consisted of three repetitions of six fruits with four wounds per fruit, per repetition for each pathogen and biocontrol agent concentration. The incidence of infections was calculated as infected wounds per total wounds after 5, 7 and 9 days of pathogen inoculation.

2.3. Dose-response experiments on pear flowers

The effect of *E. amylovora* EPS101 and EPS62e on the incidence of infections in pear flowers was assessed at the same concentrations than in fruits. Dose-response assays were done on two cultivars: Conference and Doyenne du Comice. Flowers were obtained as described in *section 4.1* from *Chapter 3* (*Figure 4.2)*, and the application of EPS62e was performed by depositing 10 µL of the antagonistic suspension in the hypanthium of each flower instead of spraying flowers with the antagonistic suspension. The experimental design consisted of three repetitions of 8 flowers per repetition for each pathogen and biocontrol agent concentration. The incidence of infections was calculated as infected flowers per total flowers inoculated and assessed after 5, 7 and 9 days of inoculation.

Figure 4.1. Wounded immature pear fruits (cv. Passe Crassane) treated with 20 µL of EPS62e suspension at 10^8 cfu·mL^{-1} and inoculated 24 h later with 10 µL of *E. amylovora* CUCM273 suspension at 10^7 cfu·mL^{-1}, after 9 days of incubation at 22 ºC, high relative humidity and 16 h of light.

Figure 4.2. Pear flowers (cv. Doyenne du Comice) treated with 10 µL of EPS62e suspension at 10^8 cfu·mL^{-1} and inoculated 24 h later with 10 µL of *E. amylovora* CUCM273 suspension at 10^7 cfu·mL^{-1}, after 5 days of incubation at 22 ºC, high relative humidity and 16 h of light.

2.4. Data analysis

Data of incidence of infections for each pathogen-biocontrol agent dose combination were used to estimate efficiency parameters for biocontrol agent and pathogen using the hyperbolic saturation model (Montesinos and Bonaterra, 1996). Equation corresponding to the hyperbolic saturation model is as follows:

$$y = Y_{max} \frac{x(1 - I)}{x(1 - I) + K_x}$$

where Y_{max} is the maximum disease proportion the pathogen can produce, K_x is a half-saturation constant corresponding to the pathogen concentration producing half the maximum disease proportion, x is the pathogen density, and I is the proportion of the pathogen inactivated by the biocontrol agent concentration. The proportion of inactivated pathogen (I) depends on the biocontrol agent concentration (z) according to the following equation:

$$I = I_{max} \frac{z}{z + K_z}$$

where I_{max} is the maximum proportion of pathogen the biocontrol agent can inactivate and K_z is the concentration of biocontrol agent that produces an inactivation of $I_{max}/2$.

The model provides parameters such as the ED_{50} of the pathogen (K_x) and the efficiency of the biocontrol agent calculated as the ED_{50} biocontrol agent/pathogen ration (K_z/K_x) (Montesinos and Bonaterra, 1996). Regression and parameter estimation were performed by means of the Gauss-Newton non-linear regression method using the JMP Statistical Package for Machintosh (JMP 2.0; SAS Institute Inc., Cary NY, USA).

3. Putative biocontrol mechanisms of EPS62e against *E. amylovora*

Determination of putative biocontrol mechanisms developed by *P. fluorescens* EPS62e against *E. amylovora* was based on growth inhibition or disease inhibition. The objective was to reject or confirm the involvement of potential mechanisms like antibiosis, nutrient competition, direct interaction, systemic acquired resistance activation and colonization of plant surfaces.

3.1. Target strain

To perform the studies of interaction between *P. fluorescens* EPS62e and *E. amylovora*, the strain EPS101 was used. EPS101 was selected because was isolated in our region, and present the common characteristics of *E. amylovora* species and high virulence on immature pear fruits**.** A spontaneous mutant of EPS62e resistant to 50 $\mu g \cdot mL^{-1}$ of nalidixic acid and another of EPS101 resistant to 100 $\mu g \cdot mL^{-1}$ of rifampicin were used in these studies. The strains retain phenotypical characteristics and performance of the parental strains. The use

of antibiotic resistant mutants allow them to be counter-select in selective culture media supplemented with the antibiotics and therefore it is possible to carry out studies of traceability in mixed populations.

3.2. Antibiosis

The involvement of antibiosis was evaluated by means of the determination of the capacity of EPS62e to inhibit several bacterial phytopathogens on different agar media, the activity of spent growth medium to inhibit *E. amylovora*, and finally dual culture separated through a semi-permeable membrane.

3.2.1. Inhibition in mixed cultures

Inhibition of EPS101 when cultured jointly with EPS62e in three different media and when cultured in EPS62e culture supernatant was determined.

Dual culture in nutrient and pear juice broths

The effect of EPS62e on growth of *E. amylovora* EPS101 was determined in LB, GA and 10% immature pear juice (v/v). Pear juice was prepared using immature Passe Crassane fruits surface disinfected and homogenized using a warring blender. Then, concentrated juice was centrifuged at 4,000 rpm for 5 min and diluted to 10% in sterile distilled water. Diluted juice was filter-sterilized through a 0.45 µm pore filter (Millicell-CM, Millipore, Bedford, MA, USA). Juice was stored at –80 ºC until use. Erlenmeyer flasks (250 mL) containing 100 mL of media were inoculated with 1 mL of an EPS62e overnight culture made on LB (~10^9 cfu·mL^{-1}) and with 1 mL of EPS101 prepared in the same form. A non-treated control inoculated with EPS101 was included for each assessed media broth. Each treatment was repeated three times. Population levels of EPS62e and EPS101 were determined after 0, 4, 8, 12, 24, 48 and 72 h of incubation on a rotatory shaker at 25 ºC. To determine population levels, 1 mL of each culture was ten-fold serially diluted and 100 µL of appropriate dilutions were spread on LB plates amended with either 50 µg·mL^{-1} of nalidixic acid or 100 µg·mL^{-1} of rifampicin to select EPS62e or EPS101, respectively. Colony counts were determined after 24 h of incubation at 25 ºC.

ANOVA was performed to test the effect of EPS62e on growth of EPS101 in the different media assessed at 72 h of incubation. Means were separated by Tukey's test ($P \leq 0.05$). The statistical analysis was performed using the SAS (Version 8.2, SAS Institute Inc., NC, USA).

Effect of EPS62e culture supernatants

The capacity of EPS101 to grow in spent supernatant of EPS62e previously grown in GA broth was determined. The medium (20 mL) was inoculated with 1 mL of an EPS62e suspension at 10^9 cfu·mL^{-1} and incubated on a rotatory shaker at 25 ºC for 48 h. Then, the cultures were centrifuged and the supernatant filtered through a 0.2 µm pore filter. From the

original culture filtrate, three fractions were prepared. One fraction was maintained without modification consisting of the original culture filtrate. The second fraction consisted of the culture filtrate amended with the half part of GA components (*Annex 2*). The third fraction consisted of the culture filtrate amended with the complete GA components. Growth of EPS101 was evaluated in the three fractions using the Bioscreen system. A suspension of 20 µL of EPS101 at 10^8 cfu·mL^{-1} was transferred to wells of a microtiter plate containing 180 µL/well of the corresponding fractions. Controls consisting of fresh GA and LB broth inoculated with 20 µL of a suspension of EPS101 to 10^8 cfu·mL^{-1} were included. Each treatment was repeated three times. The filled plates were placed in the Bioscreen for analysis (Bioscreen C, Thermo Labsystem, Finland). Measurements were taken at 600 nm, with preshaking at medium intensity for 10s prior to OD reading, and at an incubation temperature of 25 ºC. Growth was measured every 30 min during 48 h.

ANOVA was performed to test the effect of EPS62e culture supernatant on the growth of EPS101. Means were separated by Tukey's test ($P \leq 0.05$). The statistical analysis was performed using the SAS (Version 8.2, SAS Institute Inc., NC, USA).

3.2.2. Dual culture in separated compartments with a semi-permeable membrane

The inhibition of *E. amylovora* EPS101 in 10% immature pear juice by EPS62e through a semi-permeable membrane according to the method described by Janisiewicz *et al*. (2000) was used. The test was done in tissue culture plates with 24 wells per plate (Costar, Corning Inc., Corning, NY, USA) (*Figure 4.3-A*) containing Millicell culture plate inserts (Millipore Corp., Bedford, MA, USA) as the inner compartment. The inserts consisted of a polystyrene cylinder and a hydrophilic polytetrafluoroethylene membrane (pore size 0.45 µm) attached to the bottom part of the cylinder (*Figure 4.3-B*). The bottom part of the cylinder also has spacers which, after placing the cylinder in the culture well containing a liquid, allow for free liquid movement below the membrane. Each well consisted of 0.6 mL of 10% pear juice mixed with 0.2 mL of EPS62e suspension in the outside well and 0.2 mL of EPS101 suspension at 10^7 cfu·mL^{-1} in the cylinder insert. Three treatments consisting of different EPS62e concentrations were performed (10^7, 10^8 and 10^9 cfu·mL^{-1}). A non-treated control inoculated with sterile water was included. Each treatment was repeated three times. Plates were incubated at 25 ºC for 48 h. Population levels of EPS101 were determined by plate count. Thus, 100 µl of *E. amylovora* taken from the inside of the cylinder insert were ten-fold serially diluted and 0.1 mL aliquots of appropriate dilutions were seeded on LB agar plates amended with 100 µg·mL^{-1} of rifampicin. Colony counts were performed after 24 h of incubation at 25 ºC.

ANOVA was performed to test the effect of EPS62e growth on the EPS101 population levels when they were separated by a semi-permeable membrane. Means were separated by Tukey's test ($P \leq 0.05$). The statistical analysis was performed using the SAS (Version 8.2, SAS Institute Inc., NC, USA).

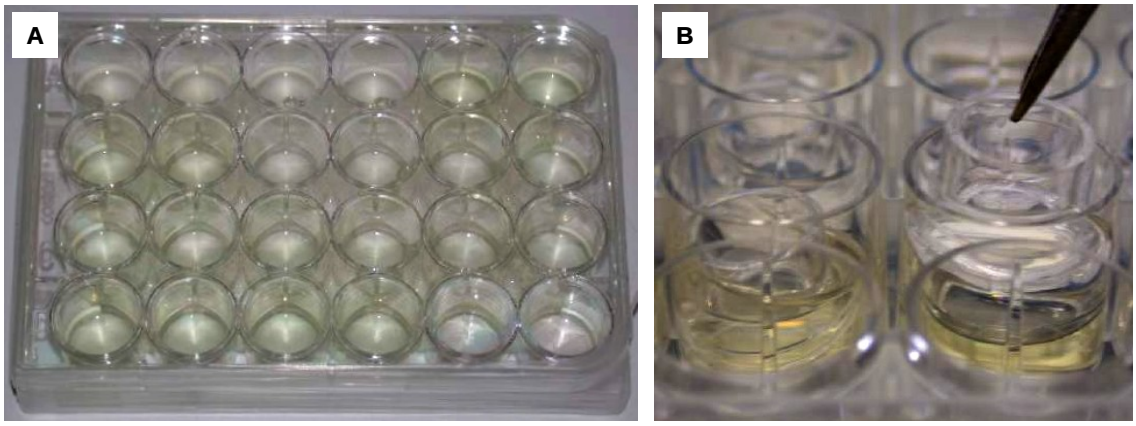

Figure 4.3. Multi-well culture plate containing pear juice (**A**) and detail of the culture plate inserts (**B**).

3.3. Nutrient competition

The involvement of nutrient competition in the biocontrol developed by *P. fluorescens* EPS62e was evaluated by means of the determination of the spectrum of nutrient use, comparison of the kinetic parameters from growth-substrate concentrations curves, and by the determination of the effect of the ratio EPS62e/EPS101 on growth of EPS101 in pear juice when both strains were separated by a semi-permeable membrane.

3.3.1. Spectrum of nutrient use and niche overlap

Nutritional profiles of carbon source utilization for EPS62e and EPS101 were determined using Biolog GN (Biolog, Inc., Hayward, California, USA) microplates according to the manufacturer's instructions. Microplates corresponding to *E. amylovora* strains were incubated for 24 h at 25 ºC. EPS62e microplates were incubated for 6 h at 25 ºC. Each well was scored as positive or negative according to the optical density at 405 nm. Wells with an optical density over 0.25 were considered positive. The niche overlapping index (*NOI*) for EPS62e and EPS101, was calculated as the number of carbon sources utilized by both bacteria respect to the total number of carbon sources utilized by either EPS62e or EPS101 (Janisiewicz, 1996).

3.3.2. Growth kinetic parameters

The maximum growth rate (μ_{max}) and the half-saturation constant for nutrient concentration (k_s), were determined for *E. amylo*vora strains CUCM273 and EPS101, and for EPS62e. The experiment was performed in LB and 10% immature pear juice. Pear juice was obtained as previously described. The kinetic parameters were estimated from concentration-growth curves, being population levels the dependent variable and concentration of nutrient the independent variable. Ten dilutions were assessed for the LB medium (1/256, 1/200, 1/128, 1/100, 1/64, 1/32, 1/16, 1/10, 1/8, 1/4), and nine for the pear juice (1/128, 1/100, 1/64,

1/32, 1/16, 1/8, 1/4, 1/2, 1). Population levels were determined using the Bioscreen system. A 10 µL suspension of the corresponding bacteria at 10^8 cfu·mL^{-1} was transferred to each well of microtiter plates containing 180 µl/well of the corresponding medium dilution. Each treatment was replicated three times. The filled plates were placed in the Bioscreen for analysis and measurements were taken at 600 nm, with preshaking at medium intensity for 10s prior to OD reading, at an incubation temperature of 25 ºC. Growth was measured every 30 minutes during 72 h.

A calibration curve was previously done for each bacterium to relate the optical density at 600 nm with the bacterial concentration. A linear regression was performed for the relation between optical density and $\log_{10}$ bacterial concentration.

Growth rates (μ) for each strain in each culture media type and the corresponding dilution were estimated by linear regression from linearized growth curves assuming an exponential growth function.

The maximum growth rate (μ_{max}) and the half-saturation constant (k_s) were estimated by linear regression using double reciprocal plot transformation of growth rate (μ) and medium concentration (S), according to the hyperbolic saturation function:

Hyperbolic saturation function: $$\mu = \mu_{\max} \frac{S}{S + K_s}$$

Linearized form: $$\frac{1}{\mu} = \frac{1}{S} \frac{K_s}{\mu_{\max}} + \frac{1}{\mu_{\max}}$$

ANOVA was performed to test if there were significant differences in K_s and μ_{max} between the *E. amylovora* strains and EPS62e. Means were separated by Tukey's test ($P \leq 0.05$). The statistical analyses were performed using the SAS (Version 8.2, SAS Institute Inc., NC, USA).

3.4. Direct interaction

The involvement of direct interaction in biological control of EPS101 by EPS62e in culture broth was evaluated. Besides, the effect of the concentrations and ratio between EPS62e and EPS101 on the degree of inhibition were also evaluated.

3.4.1. Interaction studies through a semi-permeable membrane device

The study of direct interaction was performed using the method described by Janisiewicz *et al*. (2000). The assay was performed in immature pear fruit juice prepared as previously described. Treatments consisted of different EPS62/EPS101 ratios (100:1, 10:1 and 1:1), the existence or not of direct contact between strains and pear juice concentration (1% or 10%). EPS62e was inoculated at 10^9, 10^8 and 10^7 cfu·mL^{-1} while EPS101 was inoculated at 10^7 cfu·mL^{-1}. The treatment where the strains were in direct contact consisted of 0.6 mL of pear juice in the outside well, 0.2 mL of EPS62e and 0.2 mL of EPS101 inside the cylinder insert. The treatment where the strains were separated with a semipermeable membrane consisted

of 0.6 mL of pear juice and 0.2 mL of EPS62e in the outside well and 0.2 mL of a suspension of EPS101 in the cylinder insert. A non-treated control inoculated with water instead of EPS62 was included. Each treatment was repeated three times. The plate was incubated at 25 ºC for 48 h and 100 µl of *E. amylovora* were taken from the inside and ten-fold serially diluted and 0.1 mL aliquots of appropriate dilutions were seeded on LB agar plates amended with 100 $\mu g \cdot mL^{-1}$ of rifampicin. Colony counts of EPS101 were assessed after 24 h of incubation at 25 ºC.

3.4.2. Effect of EPS62e and EPS101 cell ratio

The effect of the EPS62e/EPS101 ratio on inhibition of *E. amylovora* was studied by means of direct interaction. Different concentrations of EPS62e and EPS101 were used. EPS62e at $7.9 \cdot 10^3$ $cfu \cdot mL^{-1}$ against EPS101 at $6.3 \cdot 10^3$, $6.3 \cdot 10^4$ and $6.3 \cdot 10^5$ $cfu \cdot mL^{-1}$ to get ratios of 1:1, 1:10 and 1:100. ESP62e at $7.9 \cdot 10^4$, $7.9 \cdot 10^5$, $7.9 \cdot 10^6$, $7.9 \cdot 10^7$ $cfu \cdot mL^{-1}$ against EPS101 at $6.3 \cdot 10^3$ $cfu \cdot mL^{-1}$ to get ratios of 10:1, 100:1, 1,000:1 and 10,000:1. Treatments consisted of 0.6 mL of 10% pear juice inoculated with 0.2 mL of EPS62e and 0.2 mL of EPS101 at the corresponding concentrations. A non-treated control inoculated with water instead of EPS62e was included. Each treatment was repeated three times. The plate was incubated at 25 ºC for 48 hours and population lelvels of EPS101 were quantified as previously described.

ANOVA was performed to test the effect of pear juice concentration and direct interaction growth of EPS101. Means were separated by Tukey's test ($P \leq 0.05$). Statistical analyses were performed with the SAS (Version 8.2, SAS Institute Inc., NC, USA).

The effect of the ratio during direct interaction on *E. amylovora* was studied using a relative growth index (*RI*) that was calculated with the following equation:

$$RI = \left(\frac{(I_c - F_c)}{(I - F)} \right)$$

where, I_c is the initial population level of *E. amylovora and* F_c is the final population level on non-treated control; I is the initial population level and F is the final population level in each treatment.

The relative growth index of EPS101 was related to EPS62e:EPS101 ratio obtaining an exponential function. The rate of change (r) of relative growth rate was estimated by linear regression using natural logarithm plot transformation of relative growth (*RI*) and EPS62e/EPS101 ratio (x), according to the exponential function:

Exponential function: $RI = RI_0 \cdot \exp(r \cdot t)$

Linearization: $\ln(RI) = \ln(RI_0) + r \cdot t$

3.5. Induction of systemic acquired resistance

The induction of systemic acquired resistance was studied as a putative mechanism of fire blight control developed by EPS62e.

Self-rooted pear plants of cv. Conference (CAV clone) obtained by micropropagation (Agromillora Catalana, S.A., Barcelona, Spain) were used (*Figure 4.4*). Plants aged 2 to 3 years old, were grown in plastic pots. Plants were left outside the greenhouse during winter for chilling. During early spring, plants were pruned to leave 3 or 4 shoots and forced to bud in the greenhouse. Plants were fertilized once a week with a 200 ppm N-P-K solution (20-10-20) and were used when the shoots were about 3 or 4 cm tall and had 5 or 6 young leaves, which were the most susceptible to *E. amylovora* infection. Standard insecticide and miticide sprays were applied. Plants with two or more shoots were selected for the assay.

Figure 4.4. Self-rooted pear plants of cultivar Conference.

In a first trial the effect of leaf infiltration of EPS62e in the inhibition of *E. amylovora* EPS101 infections obtained by inoculation on wounds of young pear shoots was studied. The assay consisted of infiltration of a suspension of EPS62e into intercellular spaces of intact pear leaves with a hypodermic syringe without the needle. After infiltration, plants were incubated at 22 ºC for one week to allow induction of plant host defense responses. Then, EPS101 was inoculated following the method described by Tharaud *et al*. (1997). The youngest expanded leaves of the corresponding shoots were wounded by a double incision (~1mm) perpendicular to the midrib, approximately in the middle of the leaf. Then, one drop (10 µL) of a EPS101 suspension at 10^7 cfu·mL^{-1} was immediately placed into the wound. Inoculated plants were covered with plastic bags and incubated in an expression chamber at high relative humidity with 16 h of fluorescent light at 22 ºC. Manipulation of plants was performed in secure conditions, working in a biological safety cabinet of class II type.

Four treatments were performed consisting of two concentrations of EPS62e (10^8 and 10^9 cfu·mL^{-1}) and location of EPS101 inoculation (local and systemic). In treatments 1 and 2, EPS101 was inoculated in the same shoots where EPS62e was infiltrated (local reaction). In treatments 3 and 4, the inoculation was performed in shoots other than those infiltrated with EPS62e (systemic reaction) (*Figure 4.5*). Two non-treated controls infiltrated with water were included, one for local and another for systemic effects.

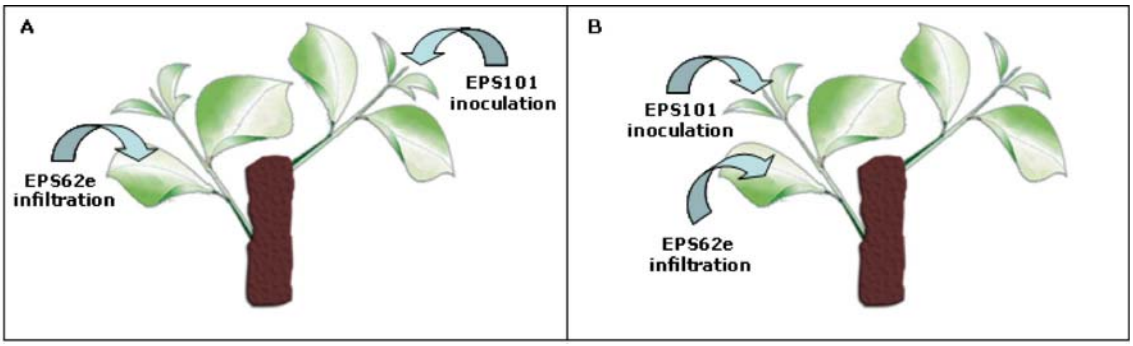

Figure 4.5. Systemic reaction (treatments 3 and 4) (**A**) and local reaction (treatments 1 and 2) (**B**).

The experimental design consisted of four repetitions of three plants per repetition for each treatment. From two to four shoots per plant were inoculated with the pathogen. Disease severity for each plant was calculated as described for the shoot blight suppression assay (*section 4.1* from *Chapter 3*). Severity evaluation was done 5 and 7 days after pathogen inoculation.

A second trial was performed following the same procedure described above but only testing the systemic reaction. EPS62e was infiltrated at 10^8 and 10^9 cfu·mL^{-1}, but EPS101 was only inoculated in shoots other than those infiltrated with EPS62e. A non-treated control infiltrated with water was included. The experimental design was the same as described above, but in this case the evaluation of severity was done 7, 9 and 12 days after pathogen inoculation.

ANOVA was performed to test the effect of EPS62e infiltrations on local and systemic disease control. Means were separated by Tukey's test ($P \leq 0.05$). Statistical analyses were performed with the SAS Package (Version 8.2, SAS Institute Inc., NC, USA).

4. Plant host colonization

The ability of EPS62e for colonization and survival on different plant organs in comparison to *E. amylovora* was evaluated. The studies were done by assessing dynamics of populations of EPS62e and *E. amylovora* on immature fruits and flowers under controlled environment conditions, scanning electron microscopy (SEM), and survival and traceability of EPS62e in pear trees under field environment conditions.

4.1. Growth kinetics on plant material under controlled environment conditions

Assays of colonization of EPS62e and EPS101 grown individually on immature pear and apple fruits and grown together on immature pear fruits and flowers were performed under controlled conditions.

4.1.1. Colonization potential of immature pear fruits

Immature pear (cv. Passe Crassane) and apple (cv. Fuji) fruits were used. Disinfected fruits (see *section 2.4* from *Chapter 2*) were wounded once with a plastic tip to a uniform depth of 5 mm and diameter of 2 mm and inoculated by deposition of 10 µL of either EPS62e or EPS101 in each wound. Four initial concentrations of EPS62e (10^5, 10^6, 10^7 and 10^8 cfu·mL^{-1}) and three of EPS101 (10^6, 10^7 and 10^8 cfu·mL^{-1}) were tested. The experimental design consisted of three repetitions of three fruits per repetition. Wounds were sampled after 0, 8, 24, 48 and 72 h of inoculation. Cells were extracted from fruits by means of homogeneization of each fruit in a sterile plastic bag with 20 mL of extraction buffer. Fruit extracts were serially diluted and 0.1 mL aliquots of appropriate dilutions were spread on LB agar plates amended with either 50 µg·mL^{-1} of nalidixic acid (EPS62e) or with 100 µg·mL^{-1} of rifampicin (EPS101). Plates were incubated at 25 ºC and colonies counted after 24 h. Population levels were expressed in cfu·wound^{-1}.

4.1.2. Dual inoculation of EPS62e and EPS101 and colonization of immature pear fruits and flowers

The ability of EPS62e to survive, colonize and inhibit growth of *E. amylovora* EPS101 in immature pear wounds and hypanthium of pear flowers when both bacteria were inoculated jointly was investigated. Passe Crassane wounded fruits (*Figure 4.6-A*) as previously described and Doyenne du Comice flowers (*Figure 4.6-B*) obtained from branches forced to bloom as described in the *section 4.1* from *Chapter 3*, were used.

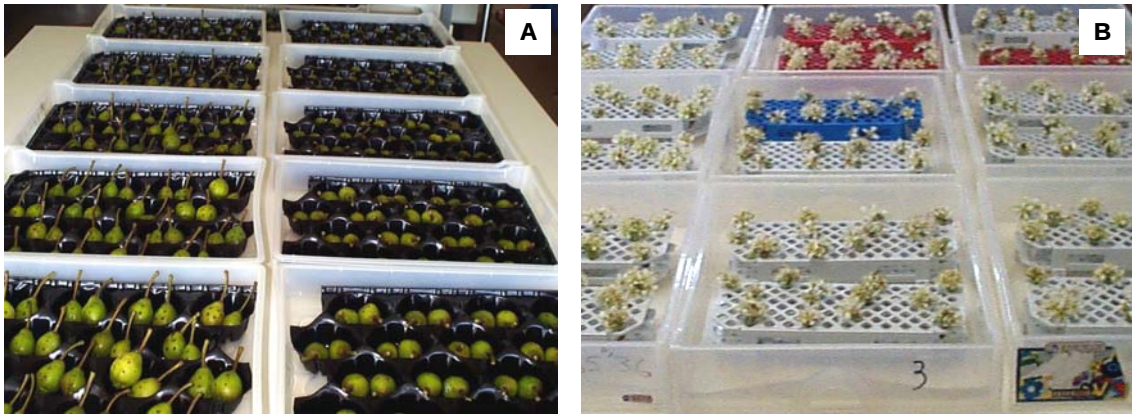

Figure 4.6. Immature pear fruits (**A**) and pear flowers (**B**) during the colonization studies.

Fruit wounds and hypanthium of flowers were inoculated with 10 µL drops of the EPS62e at 10^8 cfu·mL^{-1} 24 h before of inoculation with 10 µL drops of EPS101 at 10^7 cfu·mL^{-1}. Two controls were included, one inoculated only with EPS62e at 10^8 cfu·mL^{-1}, and another inoculated only with EPS101 at 10^7 cfu·mL^{-1}. Three repetitions of three flowers or fruits per repetition were used for each treatment. Sampling was performed at 0, 4, 15, 24, 48 and 72 or 96 h. Population levels of EPS62e and EPS101 spontaneous antibiotic resistance mutants to nalidixic acid and rifampicin, respectively, were determined as previously described and expressed in cfu·wound^{-1} and cfu·flower^{-1}, respectively.

4.2. Scanning electron microscopy (SEM)

Colonization of hypanthium of pear flowers was also studied by SEM. Pear flowers of cv. Doyenne du Comice were inoculated with 10 µL of EPS62e at 10^8 cfu·mL^{-1} and with 10 µL of EPS101 at 10^7 cfu·mL^{-1} after 24 h of EPS62 inoculation. Three controls were included, the first corresponded to a non inoculated control, the second was inoculated only with EPS62e, while the third corresponded to inoculation of EPS101 alone. After 5 days of incubation, pistils were extracted and the hypanthium was dissected by removing the stamens and petals. The hypanthium samples were immediately immersed in 2% glutaraldehyde in 0.1 M sodium-cacodylate buffer for fixation. Samples were washed in buffer and then dehydrated in an ethanol series three times (10, 30, 50, 70, 95 and 100%) and critical-point-dryed (K850, Emitech, England) using carbon dioxide as the transitional fluid. Dried material was coated with gold using a Sputter-Coater (K550, Emitech, England) and viewed in a scanning electron microscope (DSM 960A, Zeiss, Germany) at an accelerating voltage of 15 kV.

4.3. Survival and traceability of EPS62e under field conditions

The ability of EPS62e to colonize pear flowers under natural conditions was studied. Survival was evaluated in experimental plots. Besides, we tested a methodology of monitoring EPS62e based on the combination of a selectable antibiotic marker resistance and DNA fingerprinting using MRFLPs resolved by PFGE.

Pear trees in the bloom stage of cultivars 3MB-25/ML and 3MB-26 provided by the Mas Badia foundation were used. Three trees of each cultivar were sprayed with a suspension of EPS62e at 10^8 cfu·mL^{-1} (*Figure 4.7*). Each tree corresponded to a repetition. Experimental design consisted of three repetitions of three corymbs per repetition that were randomly sampled after 0, 1, 2, 5 and 10 days of treatment. Corymbs were placed individually in sterile plastic bags with 20 mL of extraction buffer and homogenized with a stomacher. Plant extracts were ten- fold serially diluted and 0.1 mL aliquots of appropriate dilutions were spread on LB agar plates amended with 50 µg·µl^{-1} of nalidixic acid. Plates were incubated at 25 ºC and colonies counted after 24 h. The population levels were expressed as cfu·flower^{-1}.

Figure 4.7. Pear trees of cultivar 3MB-25/ML in bloom stage (**A**) and detail of pear corymbs being sprayed with EPS62e (**B**).

For the evaluation of the selectable antibiotic marker resistance and MRFLP-PFGE, several resistant colonies of EPS62e recovered from LB plates amended with nalidixic acid obtained during the survival experiment were picked at random. Identification was performed by PFGE-MRFLP of genomic DNA as previously described (*section 1.3.1*).

RESULTS

1. Identification and characterization of *P. fluorescens* EPS62e

1.1. Strain identification

Cells of EPS62e are motile Gram-negative rods that produces white colonies in LB and green fluorescent colonies in KB observed under UV light (366 nm). EPS62e produces cell chains when cultured in media, like GA and minimal medium 9 (MM9).

Its metabolism is strictly aerobic, and is characterized by the following activities: Positive for catalase, β-galactosidase, arginine dihydrolase, citrate utilization, Voges-Proskauer, gelatinase, glucose acidification, melibiose acidification, arabinose acidification, gelatine, glucose assimilation, mannose assimilation, maltose assimilation, malate assimilation, gluconate assimilation, adipate assimilation, phenyl-acetate assimilation. Negative for H_2S production, urease, tryptophane deaminase, indole production, mannitol acidification, inositol acidification, sorbitol acidification, rhamnose acidification, sucrose acidification, lysine decarboxylase, ornithine decarboxylase, amygdalin acidification, reduction of nitrates to nitrites, reduction of nitrates to nitrogen, esculin, p-nitrophenyl-β-D-galactopyranoside, arabinose assimilation, mannitol assimilation, M-acetyl-glucosamine assimilation, caprate assimilation, and citrate assimilation.

EPS62e does not produce cyanide, indolacetic acid, nor chitinases. The biosynthetic genes that encode for the most common antibiotics described in *Pseudomonas* (PCA, Phl, and Prn) were not present. The strain produces siderophores on KB medium and does not present ice nucleation activity nor induce hypersensitive reaction in tobacco or geranium.

The strain was identified as *P. fluorescens* according to the Bergey's Manual of Systemic Bacteriology (Krieg and Holt, 1984) and API 20NE system. However, it was identified only at *Pseudomonas* genus level when using the API 20E system (92% confidence) and fatty acids profile (76% confidence) (*Figure 4.8*).

1.2. Characterization

1.2.1. Genomic fingerprinting using MRFLP-PFGE

The electrophoretic pattern of macrofragments of digestion of genomic DNA with *Swa* I restriction endonuclease were analyzed in several *P. fluorescens* strains (*Table 4.3*) and compared to strain EPS62e. EPS62e presents a characteristic pattern differing from other strains.

Table 4.3. Number and size of resulting fragments of the digestion of DNA of several *P. fluorescens* strains by means of restriction enzyme *Swa* I. Fragments are indicated in the range from 48 to 660 Kb. The maximum resolution of PFGE was about 12 Kb

Strain	Number of fragments	Size of fragments (Kbp)
EPS62e	12	460-477, 424-436, 363-375, 254-266, 230-242, 206-218, 181-194, 157-169, 97-109, 84-97, 60-72, 48-60
EPS818	11	620-640, 533-546, 460-472, 448-460, 375-388, 303-315, 218-230, 194-206, 157-169, 97-109, 72-84
EPS372	8	570-582, 533-546, 375-388, 327-339, 303-315, 169-181, 84-97, 48-60
BL915	11	640-660, 558-570, 436-448, 375-388, 327-339, 266-278, 218-230, 181-194, 97-109, 60-72, 48-60
CFBP2131	10	640-660, 600-620, 315-327, 291-303, 254-266, 206-218, 194-206, 121-133, 109-121, 72-84
CFBP2128	11	582-600, 485-497, 412-424, 363-375, 315-327, 218-230, 94-206, 133-145, 121-133, 84-97, 60-72
CECT378	11	600-620, 448-460, 303-315, 230-242, 206-218, 169-181, 133-145, 109-121, 84-97, 72-84, 48-60
CECT844	7	640-660, 546-558, 533-546, 206-218, 181-194, 157-169, 48-60
CHA0	8	570-582, 424-436, 327-339, 291-303, 230-242, 157-169, 84-97, 48-60

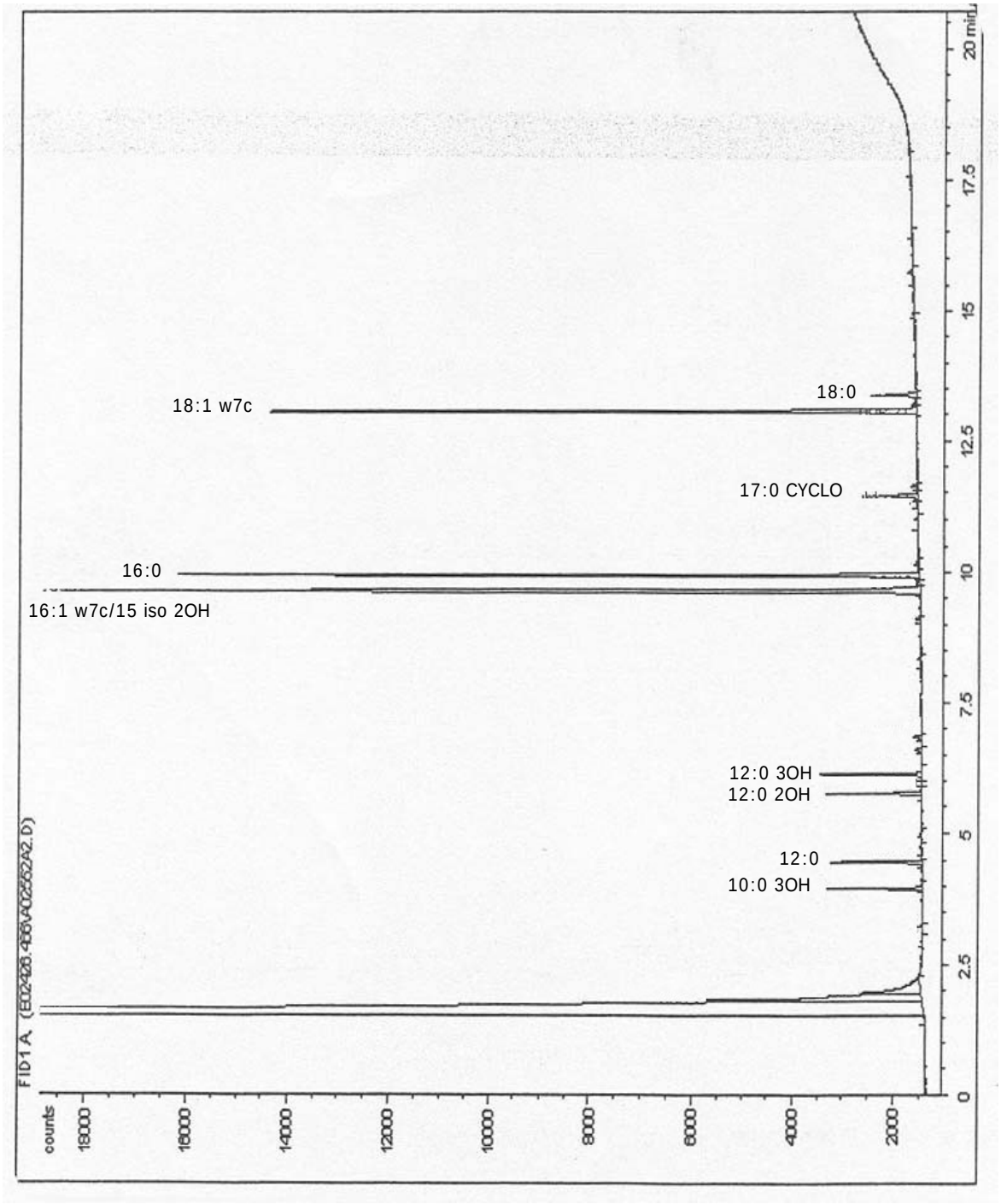

Figure 4.8. Fatty acids profile of strain EPS62e. Gas chromatogram of fatty acid methyl ester extracts of pure cultures grown on TSBA.

1.2.2. Sensitivity to chemical pesticides

EPS62e was resistant to copper hydroxide, kasugamycin and streptomycin, while it was sensible to copper sulphate, copper oxychloride and Fosetyl-aluminium, at the recommended commercial concentrations used for plant protection.

1.2.3. Spectrum of antagonistic activity in agar media

EPS62e only developed antagonism on KB medium against 7 out of the 16 strains of *E. amylovora* tested including EPS101, CFBP1430, PMV6076, UPN529, OMP-BO1185, Ea115.2 and NCPPB1819. (*Table 4.4*). In the other media assessed, there was not inhibitory activity (*Figure 4.9*). This inhibitory activity on KB was lost when iron was amended to the medium indicating that inhibition was mediated probably by siderophore production. Besides, most of the inhibited strains had been isolated from *Crataegus* sp.

Table 4.4. Antagonism developed by *P. fluorescens* EPS62e on different culture media against several strains of *E. amylovora*

Target strain	LB[x]	GA	GA-fe	KB	KB-fe
EPS100	-	-	-	-	-
EPS101	-	-	-	+	-
CUCM273	-	-	-	-	-
CFBP1430	-	-	-	+	-
PMV6076	-	-	-	+	-
UPN529	-	-	-	+	-
UPN611	-	-	-	-	-
USV1000	-	-	-	-	-
OMP-BO1185	-	-	-	+	-
IVIA1614.2	-	-	-	-	-
Ea115.2	-	-	-	+	-
EAZ4	-	-	-	-	-
NCPPB595	-	-	-	-	-
NCPPB1819	-	-	-	+	-
NCPPB2080	-	-	-	-	-
NCPPB3159	-	-	-	-	-

[x]LB, Luria Bertani; GA, glucose-asparagine; GA-fe, GA amended with iron (50 μM $FeCl_3$); KB, King's B; KB-fe, KB medium amended with iron.

For plant pathogens other than *E. amylovora*, the spectrum of antagonism of EPS62e was almost limited to the genus *Xanthomonas,* though EPS62e also inhibited *P. corrugata*, *Ralstonia solanacearum* and *P. syringae* (*Table 4.5*). Even so, in these cases, the inhibition halus was very slight with values ranging from 5 to 12 mm of diameter. *X. fragariae* and *X. campestris pv. vesicatoria* were the most inhibited species, exhibiting in KB-fe inhibition halos of 29 mm and 26 mm, respectively. KB was the medium more favorable to the

inhibition developed by EPS62e against the different bacterial indicators, though the addition of iron reduced the number of susceptible indicators to only *P. corrugata*.

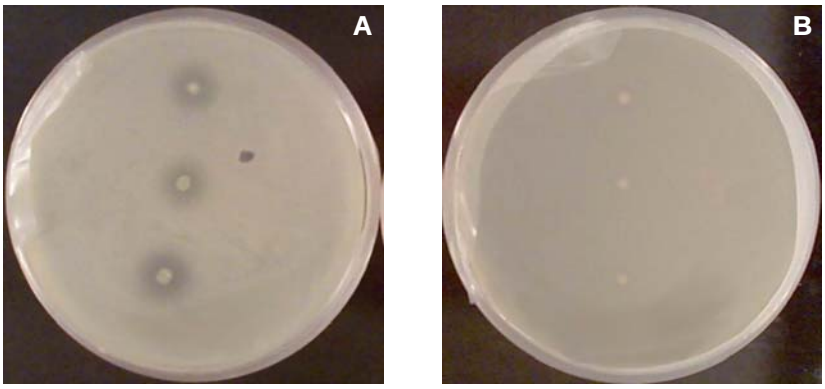

Figure 4.9. Antagonism of EPS62e against *E. amylovora* CFBP1430 (**A**) and IVIA1614.2 (**B**) in KB medium.

Table 4.5. Antagonism developed by EPS62e on different media against several phytopathogenic bacteria

Strain	LB[x]	MH	KB-fe	KB	GA	GA-fe
X. fragariae	-	-	-	+ (29)	-	-
P. corrugata	-	-	+ (12)	+ (7)	-	-
X. campestris pv. vesicatoria	+ (5)[y]	-	-	+ (26)	-	-
R. solanacearum	-	-	-	+ (7)	-	-
E. amylovora EPS101	-	-	-	+ (12)	-	-
A. tumefaciens	-	-	-	-	-	-
P. syringae pv. *phaseolicola*	-	-	-	-	-	-
P. syringae pv. *tomato*	-	-	-	-	-	-
X. arboricola	+ (13)	+(12)	-	+ (6)	-	-
P. syringae pv. *syringae*	-	-	-	+ (6)	-	-
E. amylovora EPS100	-	-	-	-	-	-
E. amylovora CUCM273	-	-	-	-	-	-

[x]LB, Luria Bertani; MH, Mueller Hinton; KB, KB; KB-fe, KB amended with iron (50 μM $FeCl_3$); GA, glucose-asparagine; GA-fe, GA amended with iron.
[y]Halus diameter in mm.

1.2.4. Spectrum of action on immature pear fruits against strains of *E. amylovora*

EPS62e inoculated at 10^8 cfu·mL^{-1} completely inhibited infections caused by all the *E. amylovora* strains in immature pear fruits after 5 days of incubation (*Figure 4.10*). Besides, when the pathogen was inoculated at 10^7 cfu·mL^{-1}, it controlled all *E. amylovora* strains with a high efficacy that ranged from 81 to 94%, except strain Ea115.2, that was not controlled.

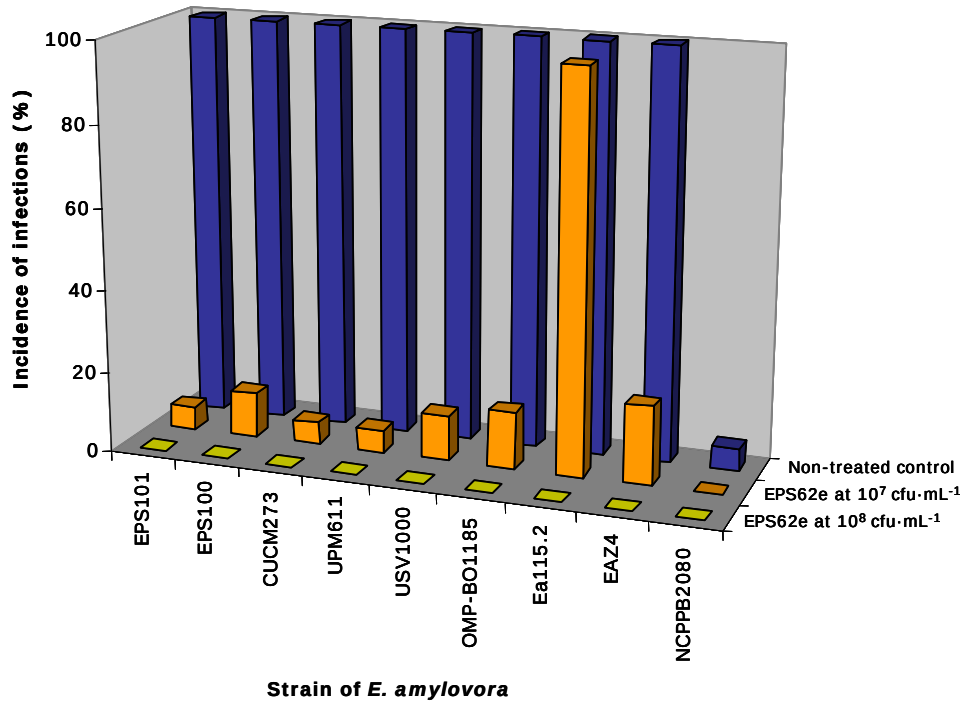

Figure 4.10. Incidence of infections in immature pear fruit treated with EPS62e at 10^7 or 10^8 cfu·mL^{-1} and inoculated 24 h later with 10 µL of several *E. amylovora* strains at 10^7 cfu·mL^{-1}. Disease was assessed after 5 days of incubation at 21 ºC and high relative humidity.

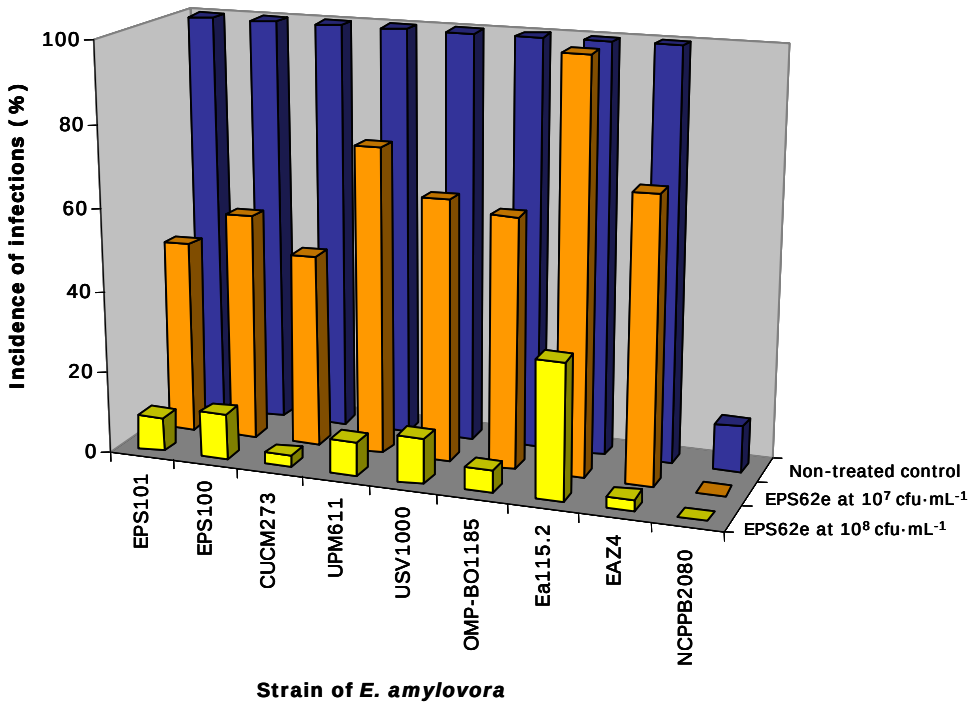

Figure 4.11. Incidence of infections in immature pear fruit treated with EPS62e at 10^7 or 10^8 cfu·mL^{-1} and inoculated 24 h later with 10 µL of several *E. amylovora* strains at 10^7 cfu·mL^{-1}. Disease was assessed after 8 days of incubation at 21 ºC and high relative humidity.

EPS62e inoculated at the higher dose controlled efficiently almost all strains of *E. amylovora* after 8 days of pathogen inoculation, with efficacy levels ranging from 89 to 97% (*Figure 4.11*). Nevertheless, EPS62e presented an efficacy of 67% in the inhibition of *E. amylovora* EA115.2. In contrast, when EPS62e was inoculated at 10^7 cfu·mL^{-1}, the levels of efficacy decreased drastically to 25-53% in most strains. EPS101 and CUCM273 were the best controlled strains, while Ea115.2 could not be controlled at 10^7 cfu·mL^{-1}.

2. Effect of pathogen and EPS62e cell concentrations on disease control

Figure 4.12 shows the effect on disease incidence levels of the treatment of wounded immature pear fruits with varying concentrations of EPS62e at different concentrations of EPS101. *Table 4.6* shows the estimated parameters of efficiency of pathogen and biocontrol agent and the goodness-of-fit according to the hyperbolic saturation model for different cultivars and plant material.

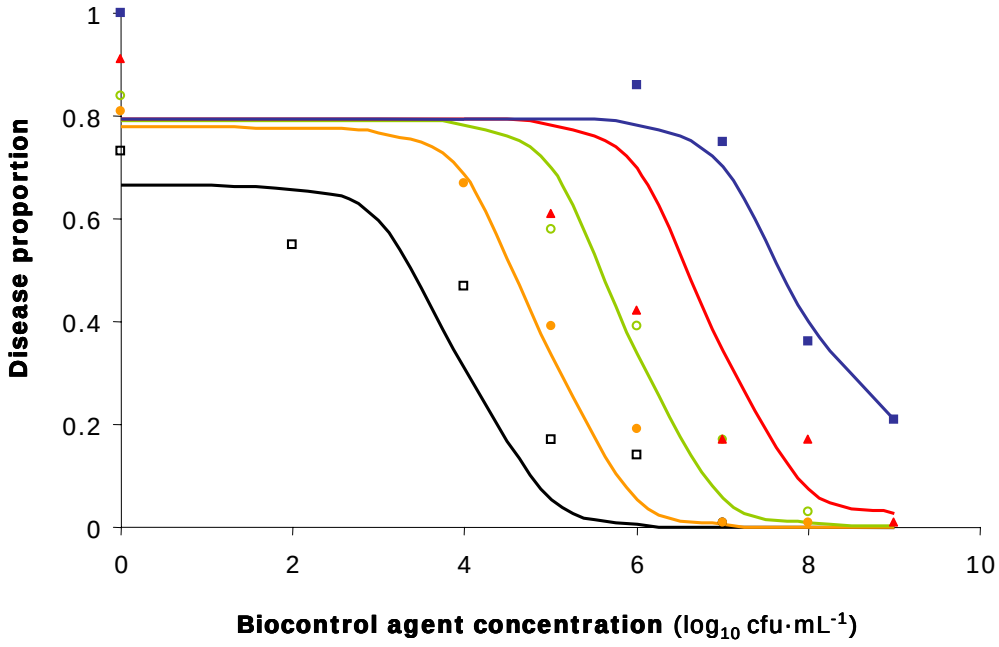

Figure 4.12. Infectivity titration of *E. amylovora* EPS101 on Passe Crassane immature fruits wounded and treated with increasing concentrations of *P. fluorescens* EPS62e. The pathogen densities were 10^4 (□), 10^5 (●), 10^6 (○), 10^7 (▲) and 10^8 cfu·mL^{-1} (■). The lines represent predictions of disease proportion at the different pathogen concentrations according to the hyperbolic saturation model, using estimated parameters shown in *table 4.6*. Disease incidence values were assessed at 21 ºC after 9 days from the pathogen inoculation.

Table 4.6. Estimated parameters and goodness-of-fit for the hyperbolic saturation model that relates the disease incidence of *E. amylovora* infections on immature pear fruits and pear flowers to the biocontrol agent and pathogen concentrations

Plant material	Pear cultivar	Parameter[x]						
		Y_{max}	K_x[y]	I_{max}	K_z	K_z/K_x	R^2	P>F
Immature fruits	Doyenne du Comice	0.74	$9.7 \cdot 10^2$	1.00	$9.2 \cdot 10^6$	$9.5 \cdot 10^3$	0.91	0.0001
	Passe Crassane	0.79	$1.9 \cdot 10^3$	0.99	$1.4 \cdot 10^3$	$7.4 \cdot 10^{-1}$	0.88	0.0001
	Conference	0.89	$4.6 \cdot 10^4$	0.76	$9.5 \cdot 10^2$	$2.1 \cdot 10^{-2}$	0.76	0.0001
	Blanquilla	0.84	$1.3 \cdot 10^4$	1.00	$5.5 \cdot 10^5$	42.3	0.92	0.0001
Flowers	Doyenne du Comice	0.77	$2.9 \cdot 10^2$	1.00	$3.1 \cdot 10^5$	$1.1 \cdot 10^3$	0.89	0.0001
	Conference	0.77	$1.5 \cdot 10^2$	1.00	$8.5 \cdot 10^4$	$5.7 \cdot 10^2$	0.79	0.0001

Data correspond to the infectivity titration of *E. amylovora* EPS101 on immature pear fruits and pear flowers of different cultivars after treatment with increasing concentrations of *P. fluorescens* EPS62e.
[x]Y_{max}, maximum disease proportion; K_x, indicates ED_{50} pathogen; I_{max}, maximum pathogen proportion inactivated; K_z, ED_{50} biocontrol agent.
[y]Densities for *E. amylovora* EPS101 and for *P. fluorescens* EPS62e are cfu·mL^{-1}.

The model reasonably fit well the data sets according to a linear relationship obtained between the observed and the predicted disease levels. Nevertheless, the model tends to give higher predicted values at high disease levels. This trend is known as fan shape effect and produces some difficulties in estimating efficiency parameters related to the pathogen and biocontrol agents such as K_x and K_z.

The maximum disease proportion (Y_{max}) estimated values ranged from 0.74 to 0.89. Although, the maximum disease proportion observed reached 1.00 for all the cultivars and plant material. Therefore, the model seems to understimate values of Y_{max}. The maximum proportion of pathogen inactivated (I_{max}) approached values of almost 1.00 in all the cultivars, independently of the plant material, except in Blanquilla immature pear fruits, that was 0.76. Thus, based on model estimations, the biocontrol agent can potentially reduce completely disease in all cultivars and plant material.

The ED_{50} of EPS101 differed among cultivars. In immature fruits the lowest value ($9.7 \cdot 10^2$ cfu·mL^{-1}) was observed in cultivar Doyenne du Comice that has been described as the most sensible cultivar to fire blight. In cultivar Passe Crassane, the ED_{50} of EPS101 reached an intermediate value, $1.9 \cdot 10^3$ cfu·mL^{-1}, while in Blanquilla and Conference the values were higher and similar, $4.6 \cdot 10^4$ and $1.3 \cdot 10^4$ cfu·mL^{-1}, respectively. In flowers, the ED_{50} values were lower than the observed in immature fruits indicating a higher susceptibility of flowers than immature fruits, reaching values of $2.9 \cdot 10^2$ cfu·mL^{-1} in Conference and $1.52 \cdot 10^2$ cfu·mL^{-1} in Doyenne du Comice pear.

The ED_{50} of EPS62e also differed among cultivars. In immature fruits ED_{50} in cultivar Blanquilla was $9.5 \cdot 10^2$ cfu·mL^{-1}, while the highest value was observed in Doyenne du Comice, with $9.2 \cdot 10^6$ cfu·mL^{-1}. For Passe Crassane and Conference cultivars, ED_{50} reached intermediate values of $1.4 \cdot 10^2$ and $5.5 \cdot 10^2$ cfu·mL^{-1}, respectively. Accordingly the sensibility to biocontrol was (from lower to higher) Passe Crassane, Conference, Blanquilla and Doyenne du Comice. Whereas, in flowers, the ED_{50} was similar for the two cultivars tested with values of $8.5 \cdot 10^4$ cfu·mL^{-1} in Conference and $3.1 \cdot 10^5$ cfu·mL^{-1} in Doyenne du Comice.

The efficiency of the biocontrol agent calculated as the ratio of ED_{50} EPS62e/EPS101 (K_z/K_x) was compared among cultivars and plant material. The highest efficiency was observed in immature fruits of Conference and Passe Crassane cultivars, with values of $2.1 \cdot 10^{-2}$ and 0.74, respectively, which indicates that in both cases less than one cell of EPS62e was needed to control one cell of EPS101. For Blanquilla cultivar, the efficiency reached intermediate values of 42.64 according to its degree of sensibility that also is intermediate. The worst efficiency was observed in flowers of Doyenne du Comice and Conference cultivars with values of $1.1 \cdot 10^3$ and $5.7 \cdot 10^2$, respectively.

3. Putative mechanisms of biocontrol of *E. amylovora* by EPS62e

3.1. Antibiosis

3.1.1. Dual culture in mixed broth

Figure 4.13 shows growth curves corresponding to EPS62e and EPS101 in mixed or separated growth in GA and LB medium or in pear juice. The maximum populations attained by EPS62e and EPS101 were similar when grew alone, with values around $2.0 \cdot 10^9$ cfu·mL^{-1} in GA, $2.6 \cdot 10^9$ cfu·mL^{-1} in LB, and $2.4 \cdot 10^8$ cfu·mL^{-1} in pear juice after 72 h. Nevertheless, the population levels of *E. amylovora* EPS101 when was co-cultured with EPS62e decreased to $3.5 \cdot 10^8$ cfu·mL^{-1} in GA medium, $1.5 \cdot 10^8$ cfu·mL^{-1} in LB medium and $3.0 \cdot 10^7$ cfu·mL^{-1} in pear juice. These population levels were significantly lower ($F=208.46$, $P<0.0001$ for GA; $F=4117.78$, $P<0.0001$ for LB, $F=2470.32$, $P<0.0001$ for pear juice) than when cultured both strains individually. The reduction of EPS101 population levels was of 0.6 $\log_{10}$ cfu·mL^{-1} in GA, of 1.2 $\log_{10}$ cfu·mL^{-1} in LB, and of 0.9 $\log_{10}$ cfu·mL^{-1} in pear juice. In contrast, growth of EPS62e was unaffected by EPS101 because the population levels attained by EPS62e cultured alone or with EPS101 did not differ significantly ($P=0.05$ in GA; $P=0.49$ in LB; $P=0.66$ in pear juice), and were $1.7 \cdot 10^9$ cfu·mL^{-1}, $2.5 \cdot 10^9$ cfu·mL^{-1} and $2.4 \cdot 10^8$ cfu·mL^{-1}, respectively.

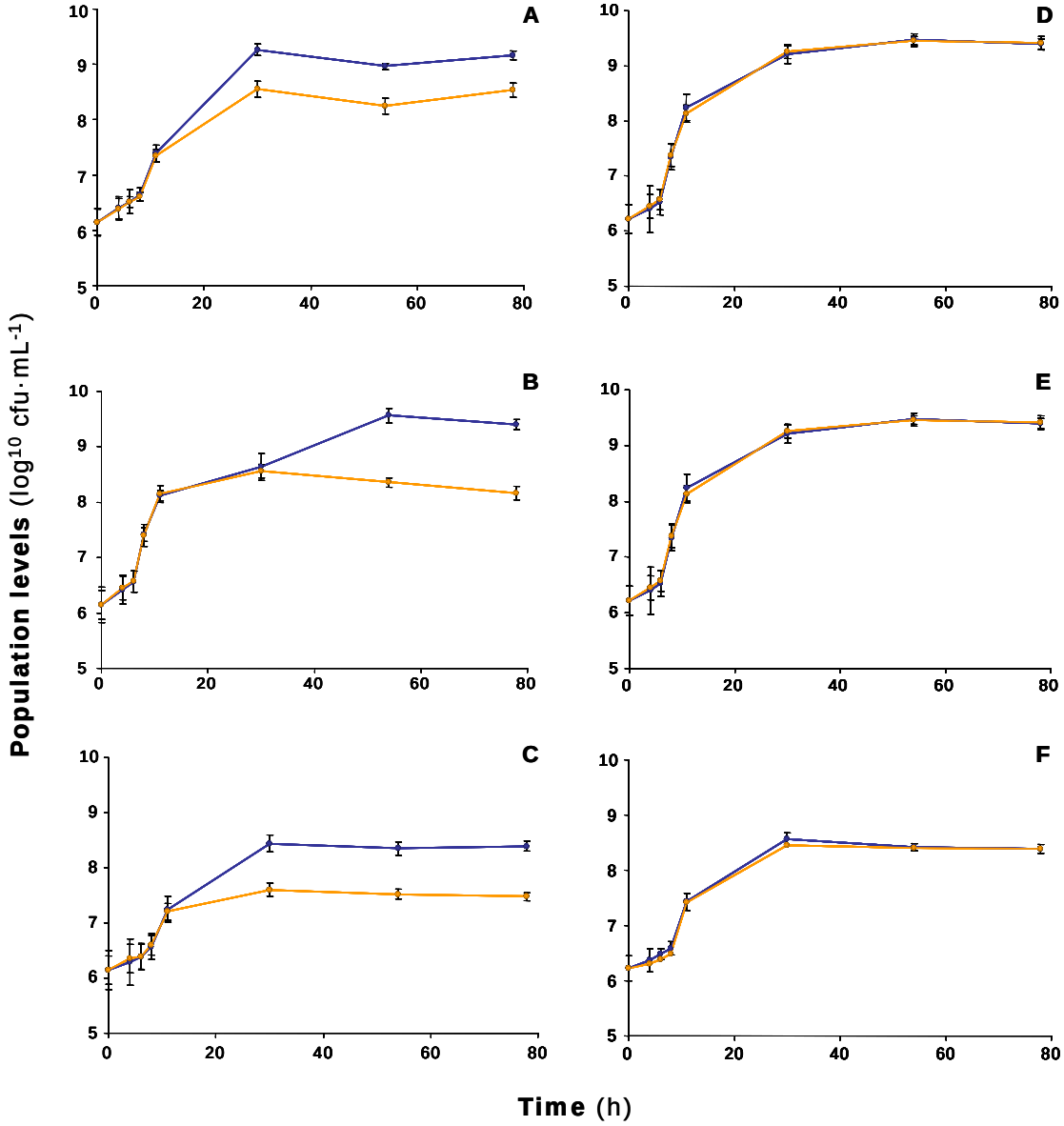

Figure 4.13. Growth curves of EPS101 grown alone (blue line) and mixed with EPS62e (yellow line) in GA (**A**), LB (**B**) and pear juice (**C**). Growth curves of EPS62e grown alone (blue line) and mixed with EPS101 (yellow line) in GA (**D**), LB (**E**) and pear juice (**F**).

E. amylovora EPS101 was unable to grow in GA culture filtrates of EPS62e, though the reconstitution of the culture filtrate with increasing amounts of GA restored growth of EPS101. When the culture filtrate was amended with 50% of GA components the growth of EPS101 was similar to the observed in fresh GA medium. Even though, when the culture filtrate was amended with 100% of the GA components, the growth of *E. amylovora* was higher than the obtained in GA medium and was similar to the obtained in LB medium (*Figure 4.14*).

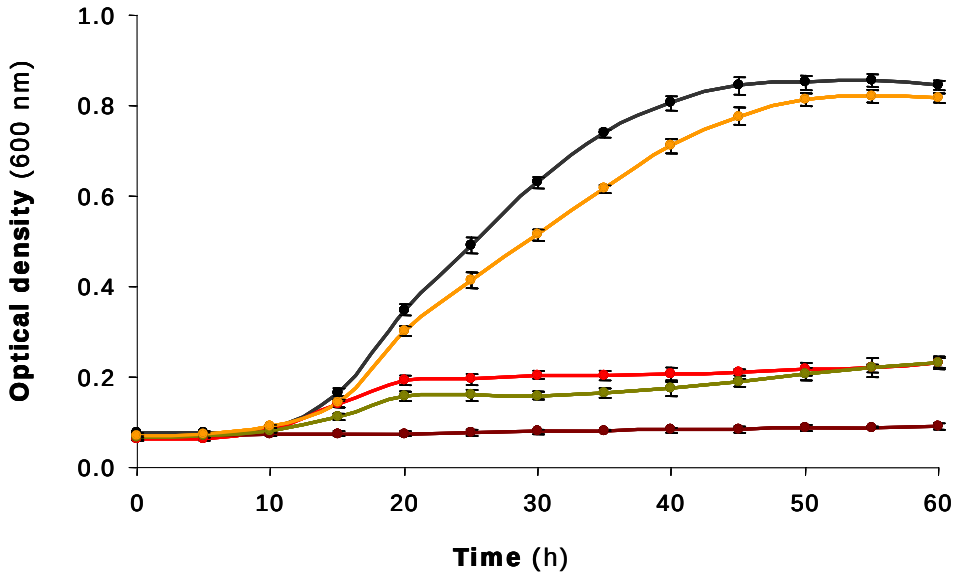

Figure 4.14. Growth curves of *E. amylovora* EPS101 grown on glucose-asparagine medium (GA) (red line), Luria Bertani (LB) (black line), GA culture filtrate of EPS62e (brown line), GA culture filtrate of EPS62e 50% reconstituted (green line), GA culture filtrate of EPS62e 100% reconstituted (orange line).

3.1.2. Dual culture in separated compartments through a semi-permeable membrane

No significant differences in population levels of EPS101 were observed when cultured alone and co-cultured with EPS62e separated with a semipermeable membrane. The results were similar for the concentrations of EPS62e of $2{\cdot}10^6$ and $2{\cdot}10^7$ cfu·mL^{-1}. Although, the treatment with 10^8 cfu·mL^{-1}, gave slightly significant lower levels of EPS101 (*Table 4.7*).

Table 4.7. Growth of *E. amylovora* EPS101 on pear juice separated by PTFE membrane cylinders from *P. fluorescens* EPS62e

Treatment[x]		***E. amylovora* population level** ($\log_{10}$ cfu·mL^{-1})
Well contents	Insert cylinder	
-	EPS101	8.56[y] a
EPS62 at $2{\cdot}10^6$ cfu·mL^{-1}	EPS101	8.60 a
EPS62 at $2{\cdot}10^7$ cfu·mL^{-1}	EPS101	8.54 a
EPS62 at $2{\cdot}10^8$ cfu·mL^{-1}	EPS101	7.87 b

[x]The experiment was performed on 10% (v/v) immature pear juice. Cylinders were separated from wells by a 0.45 µm pore size membrane filter. EPS101 was inoculated at $2{\cdot}10^6$ cfu·mL^{-1}.
[y]Values correspond to the mean of three repetitions after 48 h of incubation. Means followed by different letters are significantly different (P≤0.05) according to the Tukey's test.

3.2. Nutrient competition

3.2.1. Spectrum of nutrients used and niche overlap

The resulting niche overlapping index (*NOI*) calculated from 95 carbon sources included in the Biolog GN system was of 0.74 for EPS101 and of 0.39 for EPS62e. EPS101 utilized 27 carbon sources, and only 7 of these carbon sources were not used by EPS62e: β-Methyl D-Glucoside, Gentiobiose, Sacarose, D-sorbitol, glycil-L-Aspartic acid, Glucose-1-phosphate and Glucose-6-phosphate. EPS62e utilized 51 carbon sources, and 20 of them were utilized by EPS101: D-Fructose, Bromo succinic acid, L-alanil-Glicine, L-Aspartic acid, L-Glutamic acid, gliyil-L-Glutamic acid, Inosine, Uridine, Glicerol, N-acetyl-D-Glucosamine, D-Galactose, α-D-Glucose, D-Manitol, D-Sorbitol, D-Trehalose, methyl Piruvate, L-Proline, Mono-methyl Succinate, Succinic acid and D-Gluconic acid. This indicates that EPS62e can use most of the carbon sources used by EPS101, but not on the contrary. A total of 31 carbon sources used by EPS62e were not used by EPS101.

3.2.2. Growth kinetics

Growth curves corresponding to the pathogen and the biocontrol agent strains at several concentrations of pear juice (*Figure 4.15*) were linearized by transforming the bacterial concentrations to the natural logarithm. Growth rates (μ) for each medium dilution were estimated by linear regression using data corresponding to the exponential phase (*Table 4.8*). Curves relating growth rates with the initial medium concentration (*Figure 4.16-A*) were linearized using the double reciprocal method (*Figure 4.16-B*), and μ_{max} and K_s corresponding to each strain were estimated by linear regression (*Table 4.9*).

Table 4.8. Growth rates (μ) of *P. fluorescens* EPS62e and *E. amylovora* EPS101 and CUCM273 in pear juice at different dilutions

Dilution factor	EPS62e		EPS101		CUCM273	
	μ[x]	**CI**	**μ**	**CI**	**μ**	**CI**
1/128	0.091	0.021	0.042	0.001	0.063	0.006
1/100	0.095	0.008	0.056	0.002	0.058	0.005
1/64	0.128	0.004	0.051	0.005	0.057	0.009
1/32	0.181	0.006	0.057	0.008	0.062	0.009
1/16	0.244	0.005	0.078	0.004	0.082	0.005
1/8	0.295	0.001	0.121	0.001	0.133	0.002
1/5	0.339	0.021	0.170	0.013	0.161	0.002
1/4	0.345	0.012	0.184	0.002	0.191	0.003
1/2	0.354	0.011	0.223	0.019	0.189	0.006
1	0.353	0.012	0.218	0.022	0.182	0.007

[x]Values of μ correspond to the mean of growth rates of 3 repetitions. CI corresponds to the confidence interval for the mean.

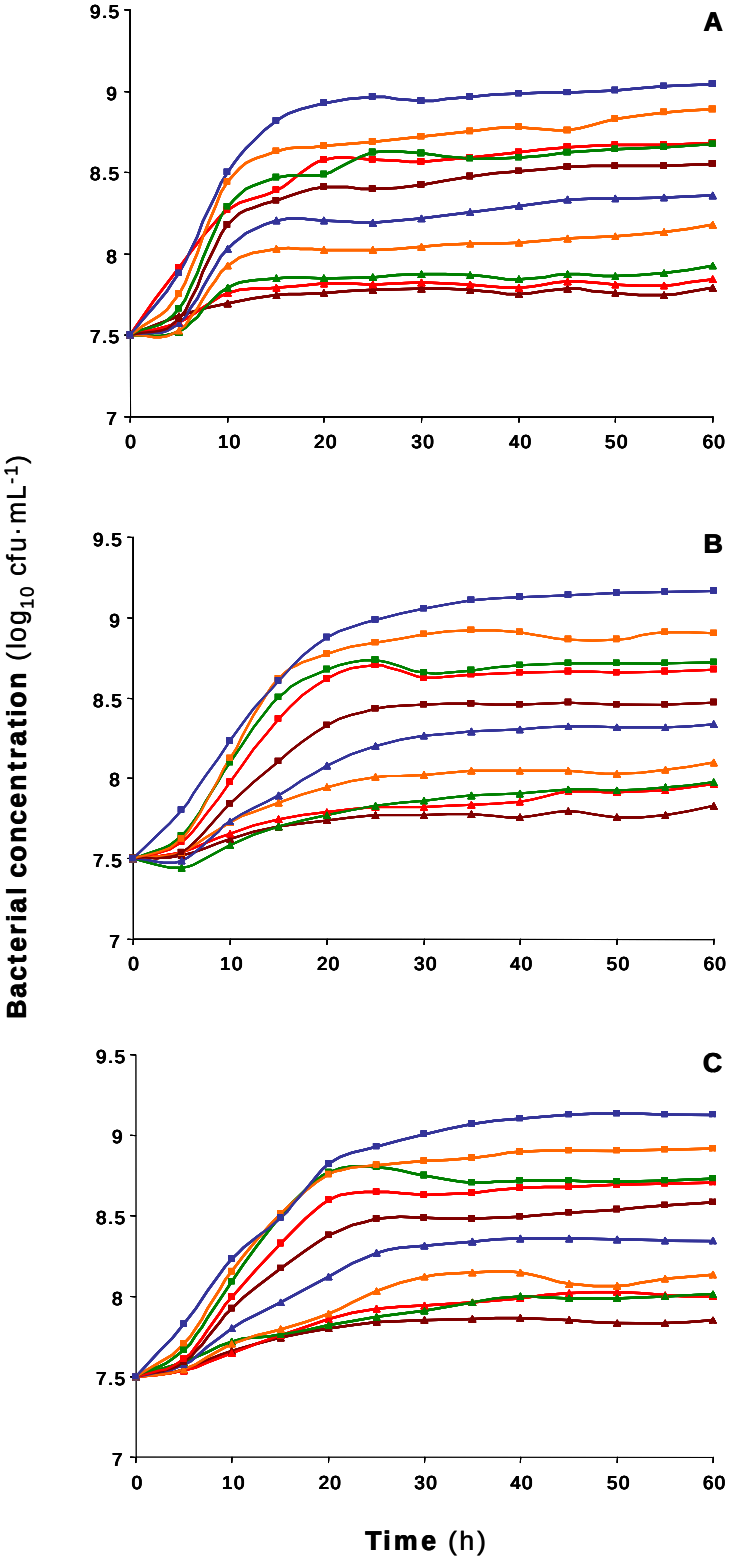

Figure 4.15. Growth of EPS62e (**A**), EPS101 (**B**) and CUCM273 (**C**) in different pear juice dilutions, 1/128 (■), 1/100 (■), 1/62 (■), 1/32 (■), 1/16 (■), 1/8 (▲), 1/5 (▲), 1/4 (▲), 1/2 (▲) and 1 (▲).

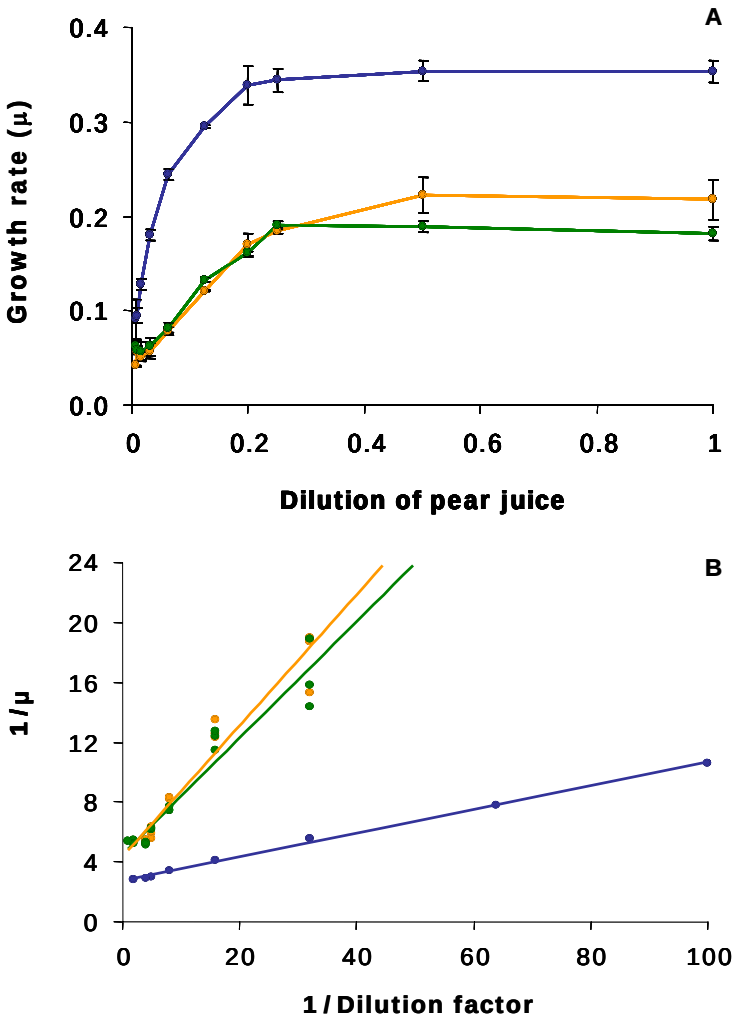

Figure 4.16. Relationships between growth rate (μ) and dilution of pear juice (**A**) for EPS62e (blue line), EPS101 (yellow line) and CUCM273 (green line), and the linear relationship obtained by double reciprocal transformation (**B**).

Table 4.9. Linear regression equations and estimated parameters (μ_{max}, K_S) obtained from linearized–growth curves of *P. fluorescens* EPS62e and *E. amylovora* EPS101 and CUCM273. Data correspond to growth rates (μ) observed with decreasing concentrations of pear juice from *Figure 4.16*

Strain	Trial	Equation[x]	R^2	μ_{max}	Mean μ_{max}	K_S	Mean K_S
EPS62e	1	y = 0.0879x + 2.6137	0.99	0.3826	0.374 a	0.0336	0.0256 a
	2	y = 0.0551x + 2.5165	0.99	0.3974		0.0219	
	3	y = 0.0622x + 2.9223	0.98	0.3422		0.0213	
EPS101	1	y = 0.4806x + 4.2681	0.97	0.2336	0.232 b	0.1130	0.0902 b
	2	y = 0.3536x + 4.8188	0.92	0.2075		0.0734	
	3	y = 0.4783x + 3.8915	0.98	0.2569		0.1232	
CUCM273	1	y = 0.3308x + 4.7955	0.90	0.2085	0.228 b	0.0689	0.1031 b
	2	y = 0.4667x + 3.9028	0.99	0.2562		0.1193	
	3	y = 0.3783x + 4.5741	0.94	0.2186		0.0827	

[x]Equation obtained by lineal regression of the apparent growth rate (μ) transformed to inverse ($1/\mu$) and the pear juice dilution factor (S) also transformed to inverse (1/S).
[y]Means followed by different letters are significantly different ($P \leq 0.05$) according to the Tukey's test.

The comparison of the strains in terms of kinetic parameters, shows that EPS62e presented a μ_{max} significantly higher (F=30.1; P<0.0001) and a K_s significantly lower (F=11.0, P=0.010) than the two *E. amylovora* strains. EPS62e presented a μ_{max} of 0.3741 and K_s of 0.0256, while the *E. amylovora* strains presented a μ_{max} around 0.2302 and K_s around 0.0967. This is an indication of a better growth potential in plant extracts and higher substrate affinity of EPS62e than the target pathogen.

3.3. Direct interaction

Growth of EPS101 was inhibited when cells of EPS62e were added in pear juice (*Table 4.10*). A significant effect of the EPS62e/EPS101 ratio and cell-to-cell contact (F=559.14, P<0.0001), pear juice concentration (F=580.08, P<0.0001) and the corresponding interaction (F=53.09, P<0.0001) was observed.

Table 4.10. Growth of *E. amylovora* EPS101 (log_{10} cfu·ml^{-1}) in immature pear juice on PFTE membrane cylinders under interaction with *P. fluorescens* EPS62e

Treatment[x]			**Growth of EPS101** (log_{10} cfu·mL^{-1})	
Well	Membrane cylinder	Ratio EPS62e:EPS101	Pear juice 1% (v/v)	Pear juice 10% (v/v)
-	EPS101	-	8.08[y] a	8.56 a
EPS62e	EPS101	1:1	8.19 a	8.60 a
EPS62e	EPS101	10:1	7.85 b	8.54 a
EPS62e	EPS101	100:1	6.39 e	7.87 b
-	EPS62E+EPS101	1:1	7.23 c	7.43 c
-	EPS62E+EPS101	10:1	6.90 d	7.23 c
-	EPS62E+EPS101	100:1	5.47 f	6.18 d

[x]The experiment was performed in well filled with 600 µL of immature pear juice inoculating 200 µL of EPS101 suspension at 10^7 cfu·mL^{-1} in the membrane cylinder, and 200 µL of EPS62e suspension at 10^7 cfu·mL^{-1}, 10^8 cfu·mL^{-1} and 10^9 cfu·mL^{-1}. Cylinders were separated from wells by a 0.45 µm pore size membrane filter.
[y]Means followed by different letters are significantly different (P≤0.05) according to the Tukey's test. Population levels were determined after 48 h of incubation at 21 ºC.

When the strains were cultured separately through a semi-permeable membrane in 10% pear juice, growth of EPS101 was not significantly affected at 1:1 and 10:1 EPS62e/EPS101 ratios, but was reduced significantly at a ratio of 100:1 (F=346.17, P<0.0001). In contrast, when the strains were cultured jointly, all the treatments had a significant effect in the reduction of growth of EPS101 in comparison to the non-treated control (without EPS62e). In addition, the level of inhibition of EPS101 increased with the ratio, with highest values at 100:1.

When bacteria were cultured in diluted pear juice (1%), significant reduction of growth of EPS101 was observed in all treatments in comparison to the non-treated control, except when EPS62e and EPS101 were cultured separately at the EPS62e/EPS101 ratio 1:1 ($F=496.06$, $P<0.0001$). Under separated or joint incubation, the inhibition of EPS101 increased with the increase of the EPS62e/EPS101 ratio.

In conclusion, inhibition of EPS101 was greater in diluted pear juice, at greater EPS62e/EPS101 ratio and when direct interaction existed between the cells of both bacteria. These results indicate that some cooperative effect of nutrient competition and direct interaction play a role in inhibition of EPS101.

Table 4.11 shows a detailed study of the effect of EPS62e/EPS101 ratio in population levels attained for *E. amylovora* EPS101 and EPS62e when cultured jointly in immature pear juice. No significant effect of the ratio was observed on the population levels attained by EPS62e that reached values around $3 \cdot 10^8$ cfu·mL^{-1}. In contrast, a significant effect of the ratio on the population levels of EPS101 was observed ($F=532.09$, $P<0.0001$). Besides, the relationship between the percentage of growth inhibition of EPS101 and the EPS62e/EPS101 ratio followed an exponential response that could be linearized by transforming the ratio to $\log_{10}$.

Table 4.11. Population levels of *P. fluorescens* EPS62e and *E. amylovora* EPS101 after 48 h of incubation at 21 ºC and relative growth of EPS101 during co-cultivation experiments at different EPS62e:EPS101 ratios

Ratio EPS62e:EPS101	**EPS62e**		**EPS101**		**Relative growth of EPS101**[z]
	Initial population ($\log_{10}$ cfu/mL^{-1})	**Final population** ($\log_{10}$ cfu/mL^{-1})	**Initial population** ($\log_{10}$ cfu/mL^{-1})	**Final population**[y] ($\log_{10}$ cfu/mL^{-1})	
individually	3.9	8.5 n.s.[x]	3.8	8.9 a	-
1:100	3.9	8.3 n.s.	5.8	8.9 a	1
1:10	3.9	8.4 n.s.	4.8	8.3 b	$2.51 \cdot 10^{-1}$
1:1	3.9	8.4 n.s.	3.8	7.5 c	$3.98 \cdot 10^{-2}$
10:1	4.9	8.4 n.s.	3.8	7.1 d	$1.58 \cdot 10^{-2}$
100:1	5.9	8.4 n.s.	3.8	6.6 e	$5.01 \cdot 10^{-3}$
1000:1	6.9	8.5 n.s.	3.8	6.2 f	$1.98 \cdot 10^{-3}$
10000:1	7.9	8.5 n.s.	3.8	5.4 g	$3.09 \cdot 10^{-4}$

[x]n.s. indicates non significant differences.
[y]Means followed by different letters are significantly different ($P \leq 0.05$) according to the Tukey's test.
[z]The relative growth was assessed as the percentage of growth of *E. amylovora* inhibited in relation the maximum growth attainable. Population levels were no log transformed.

Relative growth of EPS101 changed with the increase of EPS62e/EPS101 ratio following an exponential function (*Figure 4.17-A*). The rate of change (r) of relative growth with the ratio was estimated by linear regression using natural logarithm plot transformation of relative growth (y) and EPS62e/EPS101 ratio (x) (*Figure 4.17-B*). r presented a value of -0.54, corresponding to the slope of the regression line.

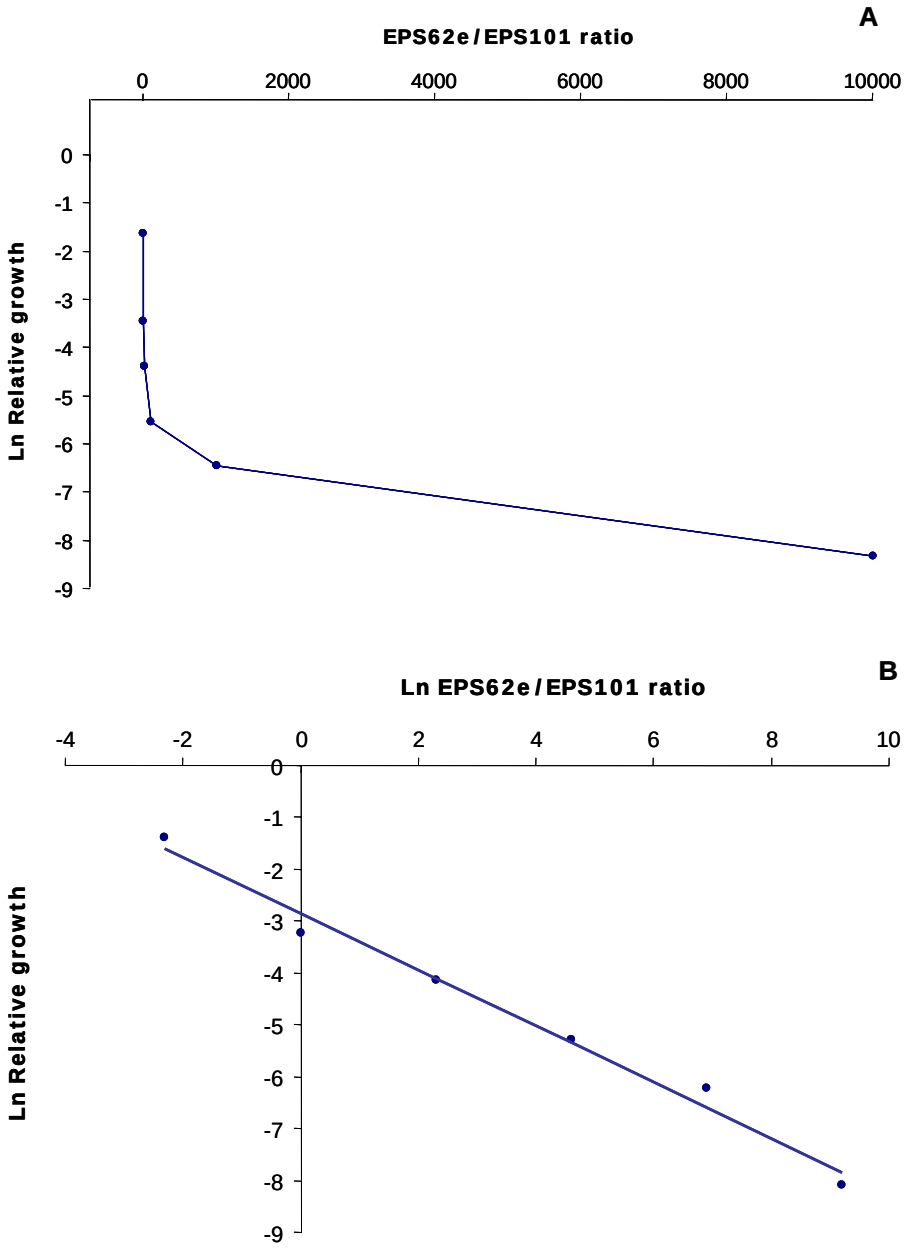

Figure 4.17. Relationship between the natural logarithm of the relative growth of EPS101 and the EPS62/EPS101 ratio when both bacteria were cultured together on 10% immature pear juice (**A**), and the linear relation obtained from linearization by transforming the ratio to natural logarithm (**B**).

3.4. Induction of systemic acquired resistance

In the first trial, a significant effect (F=5.06, P=0.01) of the infiltration of EPS62e at 10^9 cfu·ml^{-1} was observed in the reduction of severity of EPS101 infections after 5 days of pathogen inoculation. The reduction of disease severity was of 33% when EPS62e was infiltrated following the systemic procedure at 10^8 cfu·mL^{-1}, and of 60% when was infiltrated at 10^9 cfu·mL^{-1} (*Figure 4.18*). However, the effect was not significant after 7 days of pathogen inoculation. In contrast, in local reaction treatments, disease severity did not differ significantly from the non-treated control.

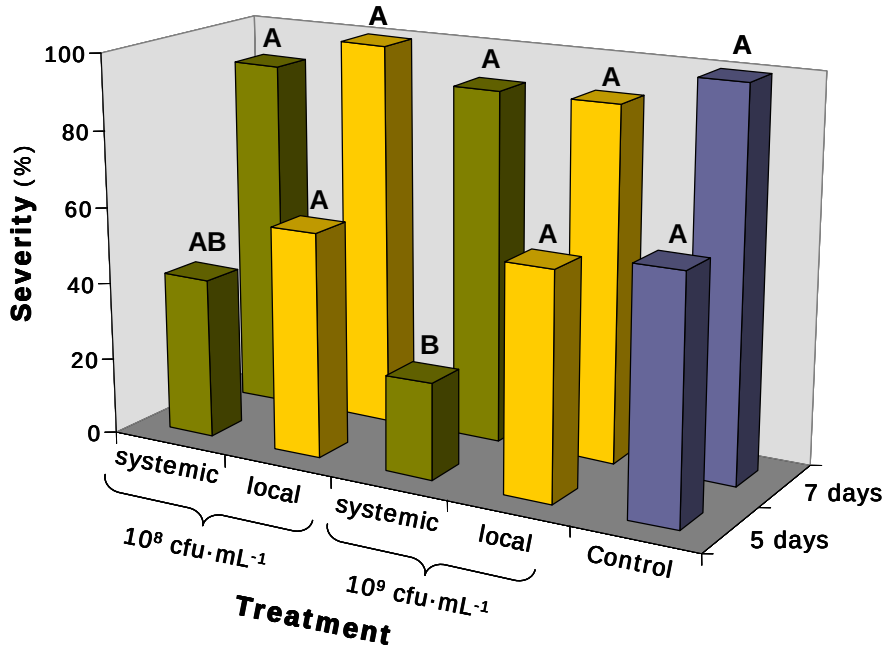

Figure 4.18. Effect of systemic (green series) and local (yellow series) applications of EPS62e on the severity of *E. amylovora* EPS101 infections on pear plants (cv. Conference) after 5 and 7 days of pathogen inoculation. Data correspond to the first trial.

In the second trial, no significant effect of the treatments of EPS62e (systemic procedure) was observed on the severity and incidence of EPS101 infections (*Table 4.12*). Even though, in this trial the severity in the non-treated control was lower than in the first trial, around 30%.

Table 4.12. Effect of treatment with EPS62e and EPS101 with a systemic strategy on disease incidence and severity in pear plants after 7, 9 and 12 days of EPS101 inoculation. Data correspond to the second trial

Treatment[x]	**Incidence** (%)			**Severity** (%)		
	7 days	9 days	12 days	7 days	9 days	12 days
Non-treated control	38 n.s.	43 n.s.	47 n.s.	23 n.s.	29 n.s.	33 n.s.
EPS62e infiltrated at 10^8 cfu·mL^{-1}	43 n.s.	42 n.s.	54 n.s.	23 n.s.	33 n.s.	47 n.s.
EPS62e infiltrated at 10^9 cfu·mL^{-1}	38 n.s.	30 n.s.	38 n.s.	19 n.s.	20 n.s.	27 n.s.

[x]The youngest leaves of shoots not infiltrated with EPS62e were wounded and inoculated immediately with 10 µl of EPS101 suspension at 10^7 cfu·mL^{-1} after one week of incubation with EPS62e infiltrated.
[y]Values correspond to the mean of incidence and severity of three replicates of three plants.
n.s. indicates non significant differences.

4. Studies of plant host colonization

4.1. Growth kinetics on plant material under controlled environment conditions

EPS101 and EPS62e showed the ability to colonize and survive in wounds produced in immature pear and apple fruits (*Figure 4.19*). EPS62e attained stable population levels of around $4.2 \cdot 10^6$ cfu·wound^{-1} in apple fruits and of $2.0 \cdot 10^7$ cfu·wound^{-1} in pear fruits after 96 h, independently of the starting population levels. However, when EPS62e was inoculated in apple wounds at 10^8 cfu·mL^{-1}, the levels attained were of $7.8 \cdot 10^6$ cfu·wound^{-1}, which were higher than those attained when starting at 10^7, 10^6 and 10^5 cfu·mL^{-1}. The population levels in pear wounds did not become stabilized for EPS101 after 72 h of incubation reaching maximum values of $1.9 \cdot 10^9$ cfu·wound^{-1} when the initial population was $2 \cdot 10^6$ cfu·wound^{-1}, and around $5.6 \cdot 10^8$ cfu·wound^{-1} at the other initial populations (10^7 and 10^6 cfu·mL^{-1}). In apple fruits, the maximum population levels observed were around $8.7 \cdot 10^6$ cfu·wound^{-1} and most of the wounds appeared healed without symptoms of exudates or necrosis, compared to pear.

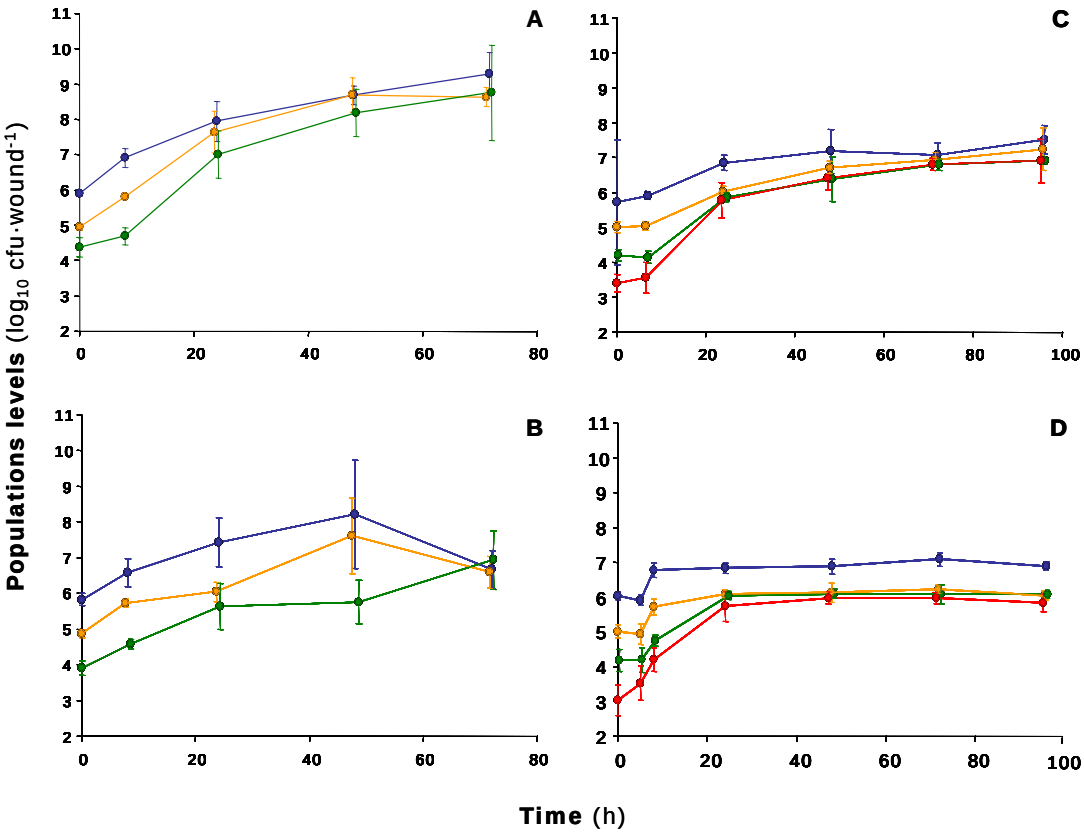

Figure 4.19. Time course of the population levels of EPS101 (left panels) and of EPS62e (right panels) on wounds of immature pear fruits (**A**, **C**) and of apple fruits (**B**, **D**). Strains were inoculated at concentrations of 10^8 (blue line), 10^7 (yellow line), 10^6 (green line) and 10^5 cfu·mL^{-1} (red line). Fruits were incubated at 21 ºC and high relative humidity. Data points correspond to the mean population levels of three replicates of three fruits and lines indicate the confidence interval for the mean.

When EPS62e and EPS101 were inoculated together in immature pear fruits (*Figure 4.20-A*), growth of EPS62e was not influenced by the presence of EPS101 because the population levels were always stable around $1.5 \cdot 10^7$ cfu·wound^{-1} after 20 h of incubation. In contrast, growth of EPS101 was strongly influenced by the presence of EPS62e, and population levels slightly decreased from the initial population ($2.3 \cdot 10^5$ cfu·wound^{-1}) reaching values of $4.3 \cdot 10^3$ cfu·wound^{-1}. However, EPS101 attained population levels around $1.6 \cdot 10^9$ cfu·wound^{-1} when was inoculated alone. Therefore, the growth potential of EPS101 was reduced by 3.9 $\log_{10}$ cfu·wound^{-1} when was inoculated together with EPS62e.

The trend observed in pear flowers was similar to the observed in pear fruits (*Figure 4.20-B*). Thus, population levels of EPS62e in flowers were similar than in fruits with values around $1.4 \cdot 10^7$ cfu·flower^{-1}. The growth of EPS101 was also influenced by the presence of EPS62e and the growth potential was reduced around 2.1 log, indicating that population levels of *E. amylovora* were reduced from $1.9 \cdot 10^8$ cfu·flower^{-1} to $1.7 \cdot 10^6$ cfu·flower^{-1}.

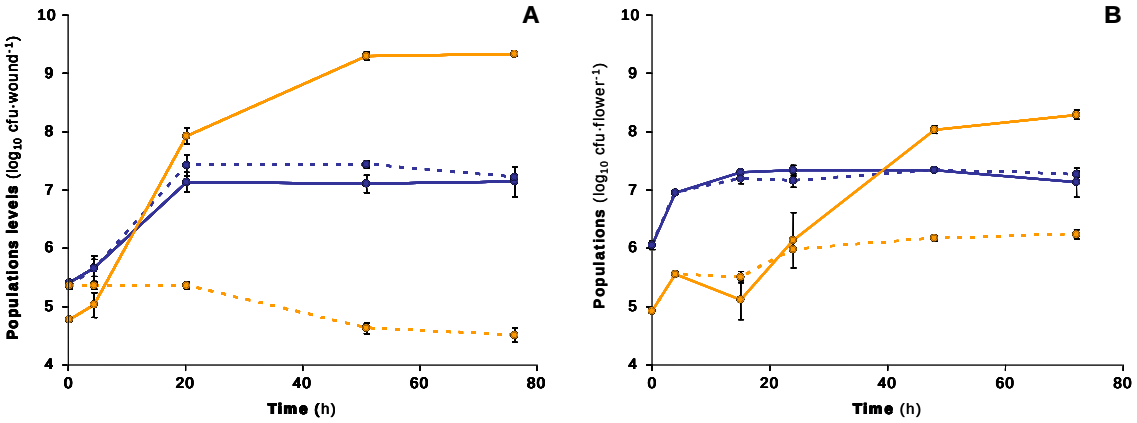

Figure 4.20. Time course of the population levels of EPS62e (continuous blue line) and EPS101 (continuous yellow line) inoculated alone, and of EPS62e (dashed blue line) and EPS101 (dashed yellow line) inoculated together on wounds of immature pear fruits (**A**) and pear flowers (**B**). EPS62e was inoculated with 10 µl of a suspension to 10^8 cfu·mL^{-1}, while EPS101 was inoculated with 10 µl a suspension to 10^7 cfu·mL^{-1} after 24 h of EPS62 inoculation. Fruits were incubated at 21 ºC and high relative humidity. Data points correspond to the mean population levels of three replicates of three fruits, bars indicate the confidence interval for the mean.

4.2. Scanning electron microscopy (SEM)

The flower surface of non-inoculated flowers remained with very low numbers of bacteria (*Figure 4.21-A*). However, in flowers inoculated with EPS101, high numbers of cells were detected after 48 h on the surface depressions between the papillae on the stigma of the flower, forming aggregates and a polysaccharide matrix (*Figure 4.21-B*). EPS62e showed a spectacular capacity to colonize the flower surface, and after 48 h of incubation under favorable conditions, the bacteria completely covered the stigmatic surface forming a dense bacterial film (*Figure 4.21-C*). When EPS101 and EPS62e grew together, both bacteria coexist on the flower surface, but at lower density. EPS62e did not cover completely the stigma surface, and EPS101 did not form cellular aggregation clamps (*Figure 4.21-D*).

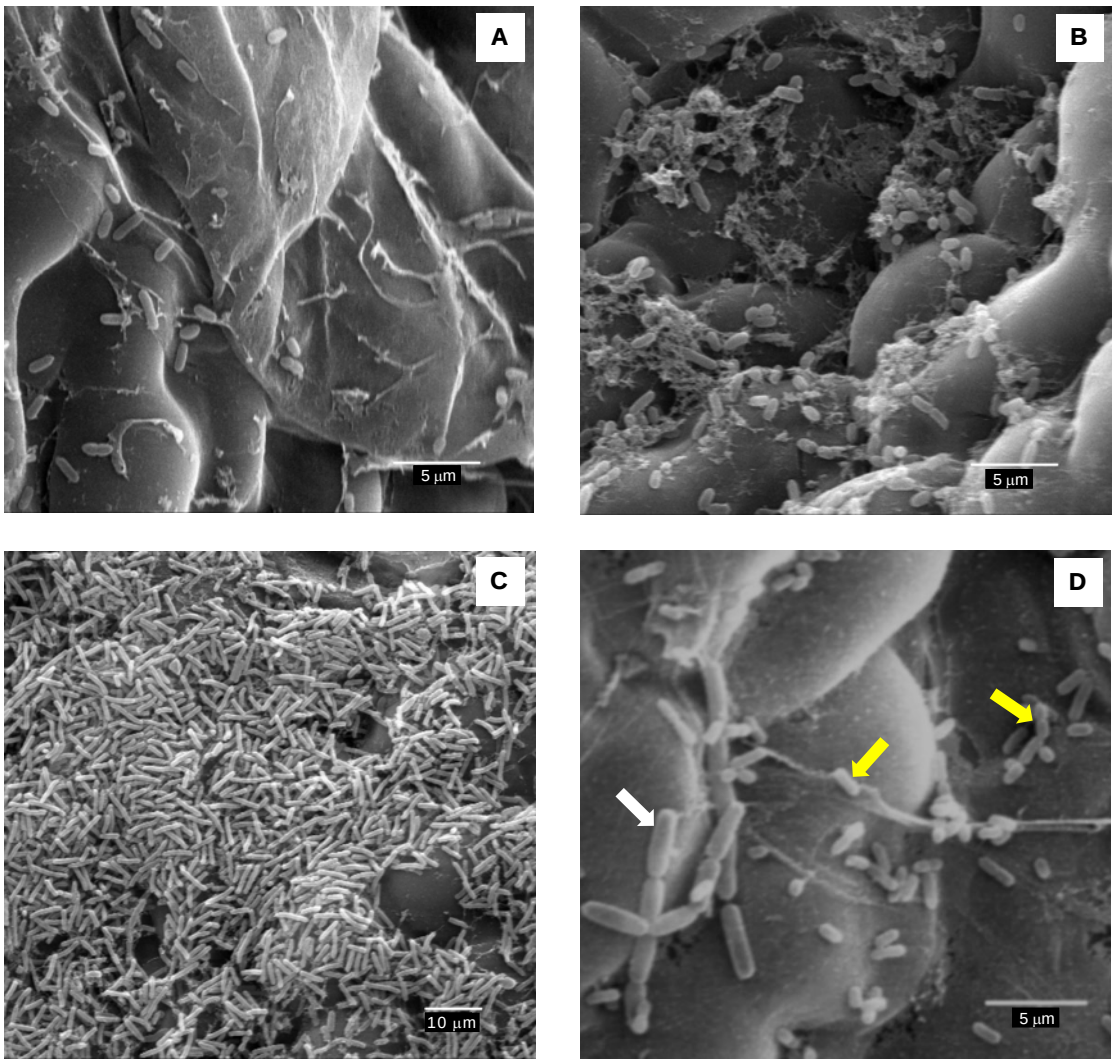

Figure 4.21. Scanning electron micrographs of cv. Doyenne du Comice pear flowers after 48 hours of incubation at 21 ºC and high relative humidity. Pictures correspond to non-treated control (**A**), inoculated with EPS101 to 10^7 cfu·mL^{-1} (**B**), treated with EPS62e to 10^8 cfu·mL^{-1} (**C**), and inoculated with EPS101 at 10^7 cfu·mL^{-1} and treated with EPS62e at 10^8 cfu·mL^{-1} (**D**). Yellow arrow indicates EPS101 and white arrow EPS62e.

4.3. Survival and traceability of EPS62e under field conditions

The population levels of EPS62e in treated pear flowers of trees under field conditions decreased upon 10 days from $8.1 \cdot 10^7$ cfu·corymb^{-1} to around $7.0 \cdot 10^5$ cfu·corymb^{-1}, independently of the cultivar (*Figure 4.22*). The decrease apparently followed two steps. A first step when the initial population decreased drastically to around $2.5 \cdot 10^6$ cfu·corymb^{-1}, and a second step in which population levels decreased slowly and became stable around $7.0 \cdot 10^5$ cfu·corymb^{-1}. Besides, these population levels were below the population levels attained by the autochthonous bacteria, which were almost stable around $5.0 \cdot 10^6$ cfu·corymb^{-1}.

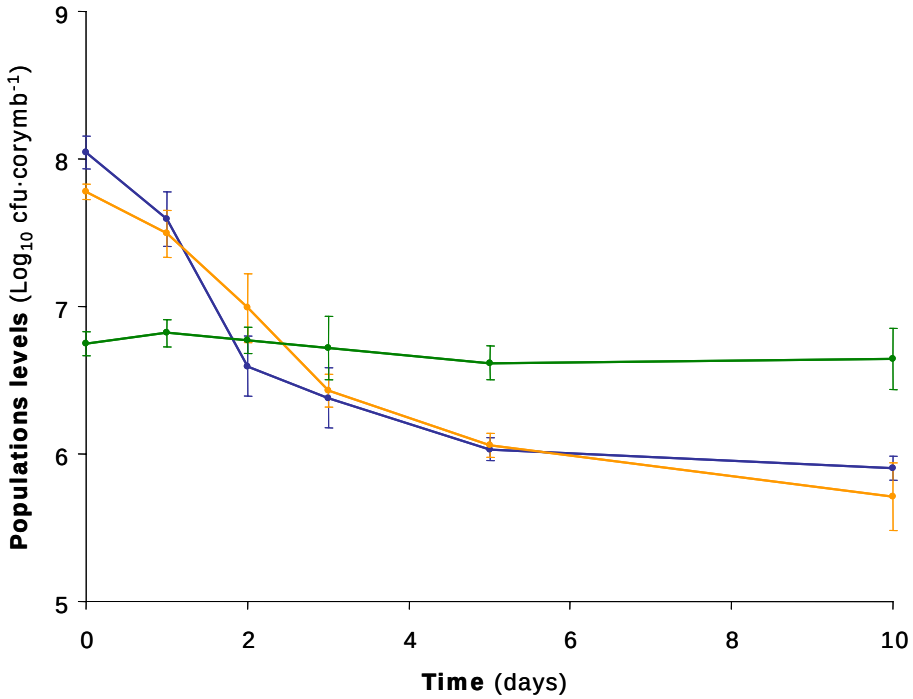

Figure 4.22. Time course of the population levels of *P. fluorescens* EPS62e on pear flowers of cultivar 3MB-25/ML (blue line) and of cultivar 3MB-26 (yellow line) under field conditions. Pear trees on bloom were sprayed to runoff with EPS62e suspension at 10^8 cfu·mL^{-1}. Green line corresponds to the autochthon base population. Data points correspond to the mean of population levels of three replicates of three fruits, and bars indicate the confidence interval for the mean.

Bacterial colonies resistant to nalidixic acid grown on Petri plates recovered from flowers after 10 days of inoculation, exhibited MRFLP-PFGE patterns which were identical to the original strain of *P. fluorescens* EPS62e applied in the treatments (*Figure 4.23*). These patterns consisted of 12 electrophoretic bands at 460-477, 424-436, 363-375, 254-266, 230-242, 206-218, 181-194, 157-169, 97-109, 84-97, 60-72 and 48-60 Kbases.

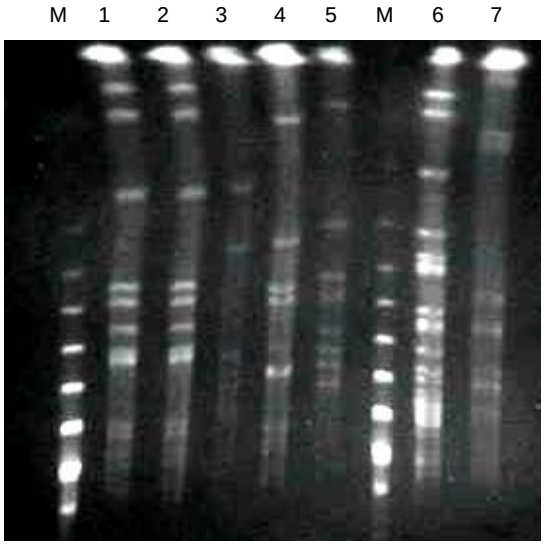

Figure 4.23. PFGE profiles of genomic DNA from various *P. fluorescens* isolates after *Swa* I digestion. The linear ramping time was 5 to 20s to 200 V. Lane 1 correspond to EPS62e in pure culture; lane 2 to a *P. fluorescens* isolate resistant to nalidixic acid recovered from treated flowers with EPS62e; lanes 3 to 7 to several *P. fluorescens* natural isolates non resistant to nalidixic acid; M, Low ranger molecular weight ladder (Low ranger, New England Biolabs Inc., Beverly, MA, USA).

DISCUSSION

1. Identification and characterization of *P. fluorescens* EPS62e

EPS62e has been selected as a potential biocontrol agent of *E. amylovora* because exhibited a high and consistent efficacy in the inhibition of infections produced on immature fruits, flowers and whole plants of pear. In addition, it has shown high efficacy in the pear cultivars assessed (Conference, Passe Crassane, and Doyenne du Comice) and against both *E. amylovora* strains tested (CUCM273, EPS101).

This strain was isolated from the surface of a pear fruit obtained from a commercial pear orchard of Conference cultivar. It was isolated using the *ex vivo* selective enrichment procedure that was based on the selection of isolates from microbiota enrichments of sample extracts that inhibited the development of *E. amylovora* infections when were applied on wounded immature pear fruits.

EPS62e was identified as *P. fluorescens* according to the Bergey's Manual of Systemic Bacteriology and API 20NE system. Cells are Gram-negative rods, motile by polar flagella, strictly aerobic, positive for catalase, oxidase and arginine dehidrolase. This strain produces diffusible fluorescent pigments under iron starvation, with typical green discoloration, grows on a wide range of temperatures and is capable of use as nutrients a broad range of substrates (Krieg and Holt, 1984).

The FAME profiling and API 20E system identified EPS62e only at the genus level as *Pseudomonas*, probably due to the fact that these are techniques mainly designed to identify animal and human pathogens and are not suitable for *Pseudomonas* species. These results agree with the fact that FAME profiling has been described by Sasser (1990) as a tool to discriminate among genera but only sometimes for species identification. Nevertheless, the main problem of FAME identification is the limited data base that is restricted to pathogenic species. This is a problem observed in other studies with *P. fluorescens* in which several strains confirmed by other methods were identified as *P. aureofaciens*, *P. putida*, *P. marginalis* or even *P. savastanoi* (Badosa, 2001). The identification based on the Biolog system has also the problem of a limited data base mainly oriented to pathogenic bacteria. In contrast, a good identification was obtained with the API 20NE system which is designed for non-enterobacteria indicating that is more suitable for *Pseudomonas* species.

EPS62e does not synthesize the most common antibiotics described for *P. fluorescens* (Phl, PCA and Prn), nor produce compounds such as cyanide, indolacetic acid or chitinases. Besides, it does not present some undesirable characteristics like INA or induction of HR.

The strain is tolerant to fungicides and insecticides, quite tolerant to copper hydroxide, kasugamycin and streptomycin, although it was sensible to copper sulphate, copper oxychloride and fosetyl-aluminium. Although, the fosetyl-aluminium have been reported with low bactericide activity with a minimal inhibitory concentration around 0.6 $g \cdot L^{-1}$ (Moragrega

et al., 1998; Ruz, 2003), EPS62e was inhibited because the concentration used was 3 $g \cdot L^{-1}$. These results agree with other studies performed with *P. fluorescens* isolates (Bonaterra, 1997) or potential biocontrol agents like *P. fluorescens* EPS288 (Frances, 2000). Therefore, EPS62e could be used in combination with other compounds allowing its use in an integrated plant protection strategy.

EPS62e presented a characteristic electrophoretic pattern of macrofragments of digestion of genomic DNA with restriction endonuclease enzyme *Swa* I which differentiate it from other isolates, type strains and biocontrol agents of the same species. The pattern in the interval from 50 to 600 Kb consisted of 12 bands of 460-477, 424-436, 363-375, 254-266, 230-242, 206-218, 181-194, 157-169, 97-109, 84-97, 60-72 and 48-60 Kbases. Molecular fingerprinting based on PFGE patterns provides highly reproducible results (Badosa, 2001) and is a useful tool to specific identification at strain level in *P. fluorescens* (Garaizar *et al*., 2000; Badosa, 2001) and *E. herbicola* (Montesinos *et al*., 2001; Montesinos *et al*., 2003; Moreno, 2003).

Therefore, PFGE pattern of EPS62e can be used as a specific method for traceability studies required for patenting and registration for commercial delivery of biocontrol agents (European Comission, 2002; Montesinos, 2003). However, determination of PFGE patterns is difficult, time consuming and cultivation dependent. Promising alternatives are DNA fingerprinting methods based in amplification of SCARs using the real time PCR (quantitative PCR) that allows at the same time specific identification and quantification without the necessity of isolation and reducing the operation time needed (De Clercq *et al.,* 2003; Montesinos, 2003; Pujol *et al*., 2004).

EPS62e showed *in vitro* antagonism against several *E. amylovora* strains and other bacterial plant pathogens on different agar media. Nevertheless, the strain lost its antagonistic activity when inorganic iron was amended to the culture media in agreement with other studies described on *P. syringae* (Bonaterra, 1997) and *S. vesicarium* (Montesinos *et al*., 1996). In these cases the antagonistic activity has been associated to siderophores production, directly by antibiotic activity or indirectly by iron exclusion. In addition, siderophores were involved in disease suppression of several pathogens by conferring a competitive advantage to the biocontrol agent under limited supply of essential trace minerals in natural habitats (Duffy and Défago, 1999) or inducing local and systemic host resistance (Leeman *et al*., 1996).

This strain presented also a wide range of activity against several strains of *E. amylovora* on immature pear fruits, because inhibited efficiently 8 out of 9 strains assessed. Although, the efficiency was hardly related to EPS62e concentration in immature pear fruits.

2. Effect of EPS62e concentration on disease control

As in other biocontrol agent-pathogen-host plant systems, the activity of EPS62e depends on the concentration of both the pathogen and the antagonist cells. Knowledge of antagonist-pathogen density relationships provides data on the population levels of the antagonist

required to achieve adequate disease control (Johnson, 1994). Dose-response models have been used as tools to determine quantitative parameters describing the efficacy of the biocontrol agent which permit comparison of different biocontrol pathosystems (Johnson, 1994; Raaijmakers *et al.,* 1995; Montesinos and Bonaterra, 1996; Smith *et al.,* 1997; Larkin and Fravel, 1990). In the present work, the median effective dose of the biocontrol agent and pathogen as well as the ratio between the median effective dose of the biocontrol agent and the pathogen (K_z/K_x) were used to determine the efficiency of EPS62e on the inhibition of infections on different cultivars on plant materials which are described that present different sensibility to *E. amylovora*. The median effective ratio (K_z/K_x) has been reported as one of the most useful parameters to compare different biocontrol agents and pathosystems (Montesinos and Bonaterra, 1996; Bonaterra *et al*., 2003). This parameter measures the efficiency of the biocontrol agent in terms of cells needed to inhibit a pathogen cell (Montesinos and Bonaterra, 1996). In the present study, dose relationships were evaluated on immature pear fruits of four cultivars (Doyenne du Comice, Passe Crassane, Blanquilla and Conference), and on pear flowers of two cultivars (Doyenne du Comice and Conference).

It was observed that all the parameters were influenced by the cultivar and plant organ. Thus, according to the median effective dose of the biocontrol agent (K_z), EPS62e was highly effective in the inhibition of *E. amylovora* infections in immature pear fruits of Passe Crassane cultivar and Conference cultivar. Besides, it showed moderately efficacy in immature pear fruits of Blanquilla cultivar and in pear flowers of Doyenne du Comice cultivar and Conference cultivar. In contrast, EPS62e showed low efficacy in immature pear fruits of Doyenne du Comice cultivar ($9.2 \cdot 10^6$ cfu·mL^{-1}). When, the median effective dose of the pathogen was evaluated, it was observed that cultivars can be grouped according to the susceptibility of immature pear fruits to EPS101 infections. The arrangement from more to less susceptible cultivar was: Doyenne du Comice cultivar, Passe Crassane cultivar, Blanquilla cultivar and Conference cultivar. These results are in accordance with the results reported by Le Lézec *et al*. (1997) in pear shoots. In pear flowers, the values of K_x were lower than in immature pear fruits, indicating that flowers are more susceptible to EPS101 infections. Besides, no differences in the susceptibility to EPS101 infections were observed between the cultivars assessed, Doyenne du Comice and Conference.

The median effective dose ratio (K_z/K_x) was used in order to compare the efficiency of EPS62e among cultivars, plant materials and other pathosystems. According to this parameter, the highest efficiency was obtained on immature pear fruits of Conference cultivar due to the fact that one cell of EPS62e was capable to inactivate twenty cells of *E. amylovora*. EPS62e was also highly effective in immature pear fruits of Passe Crassane and Blanquilla cultivars with a ratio of 1 and 40, respectively. Similar results have been reported by Mandeel and Baker (1991) in the biocontrol of *Fusarium oxysporum* f. sp. *cucumerinum* on cucumber by *F. oxysporum* C14 with a ratio of 10, and in the case of the biocontrol of *Pythium* on tomato cultivars by *Bacillus cereus* UW85 with a median effective dose ratio between 1 and 5 cfu per oospore (Smith *et al.*, 1997). In contrast, less efficiency was observed in fruits and flowers of Doyenne du Comice cultivar, with median effective dose

ratio around 10^3, and in flowers of Conference cultivar, with median effective dose ratio of 570. These values are similar to the median effective dose ratio found in the strain EPS5001 of *P. agglomerans* and in the strains EPS288 and EPS381 of *P. fluorescens* against the fungus *Stemphylium vesicarium* on pear (Montesinos and Bonaterra, 1996). Values were also similar to the strain EPS125 of *P. agglomerans* against the fungi *P. expansum* on apple (Moreno, 2003), and against *Monilia laxa* and *Rhizopus stolonifer* on stone fruits (Bonaterra *et al.*, 2003).

3. Putative biocontrol mechanisms developed by EPS62e

Knowledge of the mechanisms of action involved in the biocontrol process can permit the establishment of optimum conditions for the interaction between the pathogen and the biocontrol agent and is important for implementing biocontrol in a given pathosystem (Cook, 1993; Handelsman and Stabb, 1996).

Several mechanisms have been suggested to operate in biocontrol of plant pathogens, including antibiosis (Vanneste *et al*., 1992; Wodzinski *et al.,* 1994; Wright and Beer, 1996; Kearns and Mahanty, 1998; Stockwell *et al*., 2002), parasitism (Gaffney *et al*., 1994; Nielsen *et al*., 1998, Nielsen and Sorensen, 1999), induction of plant defence responses (Kempf *et al*., 1993), and competition for space (Lindow, 1987; Wilson *et al*., 1992; Wilson and Lindow, 1994a) and nutrients (Blakeman and Brodie, 1977; Blakeman and Fokkema, 1982; Kempf and Wolf, 1989; Chalutz and Brody, 1998). During the present work different assays were performed to make evident the implication of each one of these mechanisms on the inhibition of *E. amylovora* by EPS62e.

The antibiosis has been the most extensively studied mechanism of biocontrol due to the fact that most of biocontrol agents have been selected for their antibiotic producing activity and due to that this is the mechanism easiest to study. Determination of *in vitro* activity against bacterial indicators is a rapid and easy way to test the implication of antibiosis in the inhibition of a pathogen (Wright *et al.*, 2001). Even so, this methodology allows to confirm that the biocontrol agent produce antibiotic compounds but demonstration of the role of antibiosis is quite more laborious. Mutants lacking production of antibiotics or over-producing mutants have been used (Vanneste *et al*., 1992; Bonsall *et al*., 1997; Nowak-Thompson *et al.,* 1999). Alternatively, the use of reporter genes or probes to demonstrate production of antibiotics in the environment is becoming more commonplace (Kraus and Loper, 1995; Raaijmakers *et al*., 1997).

In the present study it was observed that EPS62e did not inhibit most of plant pathogens tested or most of *E. amylovora* strains in agar media using the agar incorporation test. Nevertheless, EPS62e inhibited some bacteria and some *E. amylovora* strains on KB medium but lost the inhibitory activity when iron was amended into the medium. This indicates that competition for iron due to the production of siderophores is probably the cause of inhibition (Montesinos *et al*., 1996; Bonaterra, 1997). Iron competition in pseudomonads has been studied and the role of siderophores produced by many *Pseudomonas* species has been

clearly demonstrated in the control of some plant diseases, either comparing the effects of purified siderophores or through the use of siderophore defective mutants (Loper and Buyer, 1991; Whipps, 2001). Therefore, the production of siderophores may play a role in the inhibition of some *E. amylovora* strains by EPS62e under iron limiting environments, like foliar or flower surfaces.

In liquid medium, *E. amylovora* EPS101 was inhibited by EPS62e when both bacteria were cultured jointly in different media (LB, GA and in pear juice). Reduction of EPS101 population levels was from 0.6 to 1.2 ten fold log decrease. Antibiosis can be one of the possible mechanisms for this effect. For this reason the capacity of *E. amylovora* EPS101 to growth in the "spent" GA medium from EPS62e according to the assay proposed for Wodzinski *et al*. (1987) was evaluated. *E. amylovora* could not grow in the spent GA medium of EPS62e, though the growth was restored when medium components were amended. Therefore, the inhibition seems to be mediated by nutrient depletion and not by antibiosis.

However, an abnormal behaviour was observed when the culture supernatant was 100% supplemented, because the growth of EPS101 was enhanced in relation to the growth obtained in fresh GA medium. This fact can be related to the production of certain nutrients or some growth factor by EPS62e that increase the growth efficiency of *E. amylovora* in this medium.

Although, in order to confirm the absence of antimicrobial compounds produced by EPS62e, the assay described by Janisiewicz *et al.* (2000) was performed. The assay consisted of a semipermeable membrane that separates the pathogen and the antagonist allowing nutrients and metabolites circulation between compartments. In this assay, EPS62e was unable to inhibit *E. amylovora* when both were physically separated, indicating that antibiotic with inhibitory activity against *E. amylovora* were not produced by EPS62e in immature pear fruit juice. Similar results have been reported in the strain EPS125 of *P. agglomerans* against *P. expansum* on apple juice (Moreno, 2003) and against *Monilia laxa* and *Rhizopus stolonifer* in nectarine peel leachate (Bonaterra *et al.*, 2003). Although, in the case of EPS62e, a significant reduction of *E. amylovora* population levels in relation to the non-treated control was observed when EPS62e was applied 100 times more concentrated than EPS101 (ratio 100:1), indicating that inhibition was probably mediated by nutrients competition. Therefore, it is unlikely that production of antibiotic substances is important because neither the cell free spent culture of EPS62e amended with GA components nor the physical separation of the biocontrol agent and pathogen by a membrane filter produced inhibition of *E. amylovora* growth. Moreover, EPS62e does not produce the most common antibiotics described on *P. fluorescens* neither inhibits most of bacterial phytopathogens nor of *E. amylovora* strains in dual culture plate assay using different media.

The competition for limited resources is another possible mechanism that could be involved in the biocontrol of *E. amylovora* by EPS62e. In previous assays, it was observed that nutrient competition was involved in the inhibition of *E. amylovora*.

The availability of nutrients is a factor governing growth of microbial populations in plant surfaces (Andrews, 1992; Wilson and Lindow, 1994b) because plant surfaces are a nutrient-limited environment, mainly in carbon and nitrogen resources (Wilson and Lindow, 1994b; Wilson *et al*., 1995). Several studies focused on the implication of nutrients competition in the biocontrol of aerial plant diseases have been reported specially against *P. syringae* and *E. amylovora* (Wilson and Lindow, 1994a; Ji and Wilson, 2002; Wilson *et al*., 2002; Dianese *et al*., 2003). Some of these studies have found a correlation between the nutritional similarity and the suppression of disease (Wilson and Lindow, 1994b; Ji and Wilson, 2002).

Nutritional similarity between *E. amylovora* and EPS62e was quantified by using *NOI* defined as the ability to utilize carbon sources not utilized by a competing strain (Wilson and Lindow, 1994b). It was observed that EPS62e presented high *NOI* respect *E. amylovora* EPS101 (0.74) which indicates that is capable to use most of carbon sources used by EPS101. Therefore EPS62e could potentially compete and exclude *E. amylovora* on leaf and flower surfaces by means of depletion of limited resources. This is in agreement with Ji and Wilson (2002) observing that suppression of bacterial speck of tomato (*P. syringae* pv. *tomato*) was correlated with nutritional similarity between the pathogenic and nonpathogenic bacteria suggesting that preemptive utilization of carbon sources was probably involved in the biological control of the disease.

Contrarily, EPS101 presented a low *NOI* respect EPS62e (0.39) which indicates that the capacity of EPS101 to exclude EPS62e from plant surface by nutrients competition is low because EPS62e can use several carbon sources that can not be used by *E. amylovora*. This fact provides the option to selectively increase the population size and biocontrol efficacy of EPS62e through nutritional amendment of limited nutrients such as it has been described by Ji and Wilson (2002) on the biocontrol of bacterial speck of tomato by *P. syringae* Cit7.

Once the potentiality of EPS62e to compete for nutrients with *E. amylovora* was evaluated by means of *NOI* analysis, growth kinetics was studied on immature pear juice. Kinetics permit to compare EPS62e and *E. amylovora* according to the maximum growth rate (μ_{max}) and the constant of medium affinity (K_s). In these studies, EPS62e presented a higher μ_{max} and lower K_s (higher affinity) than the strains CUCM273 and EPS101 of *E. amylovora*. Hence, EPS62e grows faster and presents more affinity for the medium than *E. amylovora* CUCM273 and EPS101 indicating that EPS62e exhibits a higher ability to growth on immature pear fruits, and probably on other plant parts, than *E. amylovora*.

From these results, it is concluded that EPS62e have aptitudes to outcompete *E. amylovora* in immature pear juice by means of depletion of limited nutritients and it is suggested that preemptive utilization of carbon sources of fruit wounds and flower surface is involved in the biocontrol of disease. Nevertheless, it would be necessary to confirm the role of competition on disease control, for example by means of evaluation of the efficacy of catabolic mutants on disease control with respect to the wild-type (Ji and Wilson, 2002).

Cell-to-cell interactions were also evaluated as a possible mechanism involved in the inhibition process. The method described by Janisiewicz *et al.* (2000) based on the physical

separation of cells has been used to study competition for nutrients without the interference of competition for space. Also, other mechanisms in which direct contact between the antagonist and a pathogen is not required can be studied (Bonaterra *et al*., 2003; Moreno, 2003). In the present work, this method was used to perform interaction studies between EPS62e and EPS101 at different ratios in order to determine the mechanisms of biocontrol involved in immature pear juice. These studies were specially focused on the direct interaction and for this reason the effect of EPS62e on the growth of *E. amylovora* was assessed when both bacteria were cultured together or separated at different concentrations and ratios. Inhibition of growth of *E. amylovora* was only observed when there was a direct cell-to-cell interaction, while no effect was observed when the antagonist and the pathogen cells were physically separated by a membrane filter which permits nutrient and metabolite interchange. These results agree with the results obtained in the biocontrol by *P. agglomerans* EPS125 of *Monilia laxa* and *Rhizopus stolonifer* in postharvest of stone fruits (Bonaterra *et al*., 2003) and of *P. expansum* in postharvest of pear and apple fruits (Moreno, 2003). However, some inhibition of *E. amylovora* was observed with cells separated by the membrane filter when pear juice was diluted until 1% or with the ratio EPS62e:EPS101 above 100:1. This indicates that the inhibition is probably related to nutrient availability, and therefore under some conditions the competition for nutrients can be involved in the inhibition of *E. amylovora* growth in accordance with the results previously commented.

The study of cell-to-cell interactions at increasing concentrations and ratios of EPS62e and EPS101 was performed with the aim to quantify the effect of direct interaction on the inhibition of *E. amylovora*. In order to carry out this study, both bacteria were cultured together in immature pear fruit juice at 10% at different concentration ratios EPS62e:EPS101, from 1:100 to 10,000:1. The assay was performed in fruit juice at 10% to avoid as much as possible nutrient influence and therefore the influence of competition for nutrients. Although, the effect of competition for nutrients was probably present in ratios above 100:1, more concentrated juice could not be obtained because of filtration problems to be sterilize it. This assay confirmed the implication of direct interaction on inhibition of *E. amylovora* in culture and also allowed to determine the rate of change (*r*) of relative growth of *E. amylovora* with respect to the EPS62e/EPS101 ratio providing a parameter useful to compare antagonistic strains.

Finally, the involvement of induction of plant defense response was evaluated as a possible mechanism in the inhibition of *E. amylovora* by EPS62e. Induction of plant defense responses in the biocontrol of fire blight has been hypothesized for *E. amylovora* mutants in genes involved in regulatory functions (Tharaud *et al*., 1997). In the present study, the same methodology was used to evaluate the effect of the infiltration of an EPS62e in the leaf mesophyll in the control of shoot blight. In the first trial, it was observed that systemic inoculation reduced significantly the incidence and severity of *E. amylovora* infections five days after the pathogen inoculation. This effect only produced a delay of the infection process because disease levels on these treatments were similar than the non-treated control after seven days of pathogen inoculation. However, no effect was observed in a

second trial. Possibly the problem was not due to the low consistence of the EPS62e strain but the variation in the state of development of the plants. More exhaustive studies would be necessary in order to get a convincing conclusion. Different times of stimulation should be tested in accordance with studies performed with synthetic plant defense stimulators which describe an important effect of timing of product application on disease control (Ruz, 2003).

4. Studies of colonization

The capacity to colonize and survive in different plant organs under controlled environment and field conditions was also studied. The ability to grow in the same ecological niche as the pathogen is a necessary characteristic of any biocontrol agent of fire blight. Without this ability the biocontrol agent can not interact with the pathogen and develop an efficient mechanism of inhibition (Vanneste, 1996). The establishment of the antagonist on the plant surface is a critical phase in disease control for competition with the pathogen for sites and nutrients (Stockwell *et al.*, 1998). For example, it has been demonstrated that colonization of the stigmatic surface of flowers with nonpathogenic bacteria such as *P. fluorescens* or *Pantoea agglomerans* can greatly inhibit colonization by *E. amylovora*, leading to a substantial reduction in disease (Lindow *et al.,* 1996; Johnson and Stockwell, 1998; Pusey, 2002). For this reason, the capacity of EPS62e to colonize and survive on plant tissues was evaluated.

In individual inoculations in wounds in immature apple and pear fruits under controlled environmental conditions, EPS62e and EPS101 colonized and quickly multiplied until the carrying capacity of the wounds was reached (around $5 \cdot 10^7$ cfu·wound^{-1}). Nevertheless, in pear fruits, the population levels of EPS101 surpassed the carrying capacity of the wounds ($3.8 \cdot 10^{10}$ cfu·wound^{-1}) probably due to exudate production. In apple fruits this was not observed. This also may be the reason of the high variability observed in the population levels attained in apple.

In contrast, the growth rate and population levels of EPS101 were significantly reduced in wounds and flowers when EPS62e was previously inoculated (preemptive colonization). The ability of EPS62e to colonize and multiply on flowers surface was confirmed by scanning electron microscopy, showing EPS62e recovering the entire stigmatic surface in only 24 h. Nevertheless, EPS101 cells produced aggregates on a polysaccharide matrix that probably confers them adherence and some protection to aerial stresses (water availability, UV radiations). When both bacteria were sequentially inoculated, the number of cells of *E. amylovora* were drastically reduced from the flower surface with the disappearance of cell aggregates.

All these results indicate that EPS62e have the ability to colonize and grow in fruit wounds and flowers and that pre-emptive colonization is affecting *E. amylovora* inhibition. This agree with the results obtained in *P. fluorescens* A506 that does not develop antibiosis and the inhibition has been related to competition for nutrients and space (Lindow *et al.*, 1996). Therefore, the inhibition of *E. amylovora* on flowers, and probably in immature fruits and

shoots could be due to the combination of colonization ability with other mechanisms such as the competition for nutrients and direct interaction between the antagonist and the pathogen.

EPS62e exhibits also the capacity to colonize and survive under field conditions in flowers of two autochthon pear cultivars. The assay was performed in an experimental plot I spring time at about full bloom. Although, the population levels decreased and remained stable around $7 \cdot 10^5$ cfu·corymb^{-1}. Nevertheless, the final population level of EPS62e was below the carrying capacity of flowers that was around $5 \cdot 10^6$ cuf·flower^{-1}. Probably, this is because flowers were already colonized by indigenous bacteria and EPS62e cells could not established because the entire flower surface was covered by indigenous bacteria. This is contrarily to what happens under controlled environment conditions. This also agree with studies based in scanning electron microscopy (SEM) describing the heterogeneity of bacteria distribution on leaf surfaces which limit the available surface to colonize (Leben, C. 1981; Leben, 1988; Wislon *et al*., 1999). Thus, EPS62e can not displace the indigenous bacteria and for this reason the population levels attained values lower than the allowed for the carrying capacity of the flowers. Nevertheless, EPS62e might survive in flowers probably due to the occupation of empty sites that remains in the surface. EPS62e also presents the capacity to move readily from inoculated to non-inoculated flowers (data not shown) in accordance with other studies that describe the ability of some strains to colonize newly opened flowers (Johnson *et al*., 2000; Pusey, 2002). This capacity is important because it facilitate biocontrol in flowers that are closed when the biocontrol agent is applied.

Simultaneously to colonization studies, a method for monitoring EPS62e was evaluated. Several studies of biocontrol used a viable count method based on semi-selective culture media or even colony appearance, for detection and quantification of introduced strains (Benbow and Sugar, 1999; Nunes *et al*., 2002; Janisiewicz and Jeffer, 1997). However, this method may have interference from the autochthonous microbiota corresponding to the same species of the strain used as biological control agents. To avoid this problem many field studies dealing with monitoring introduced biocontrol agents used spontaneous mutants harboring a selectable antibiotic (Wilson and Lindow, 1993; Johnson, 1994; Montesinos *et al.*, 1996). Nevertheless, in the present study a monitoring methodology that consisted of a spontaneous nalidixic acid resistance combined with DNA fingerprinting based on the electrophoretic pattern of macrofragments of digestion of genomic DNA with restriction endonuclease *Swa* I (PFGE pattern) was assessed. This technique of DNA fingerprinting was selected because it is highly reproducible and allows a high discrimination to strain level when applied to *P. fluorescens* species (Badosa, 2001) and other species, like *Salmonella enterica* (Garaizar *et al.*, 2000), *Lactobacillus rhamnosus* and *L. casei* (Tynkkynen *et al.,* 1999).

The use of the PFGE technique to identify *P. fluorescens* EPS62e upon treatments confirmed that bacteria resistant to nalidixic acid recovered from corymbs pertained to *P. fluorescens* EPS62e previously introduced in treatments. Other monitoring studies used selective media to determine population levels, such as in strain CPA-2 of *P. agglomerans* (synonymous of *E.*

herbicola) (Nunes *et al.*, 2002), but this method may be not sufficiently specific to distinguish the introduced strain from autochthonous strains, because *P. agglomerans* is present as a common inhabitant on fruit surfaces (Montesinos, 2003). The combination of a nalidixic acid resistance selectable marker and DNA fingerprinting based on the PFGE pattern allowed us to distinguish EPS62e from other *P. fluorescens* that naturally colonized the fruit trees surface.

In conclusion, the biological control agent *P. fluorescens* EPS62e selected in the present study is effective at moderately concentrations in preventive treatments for control of fire blight on immature fruits, flowers and shoots of pear under controlled environment conditions. The preemptive exclusion of the pathogen by plant surface colonization and nutrients depletion, and cell-to-cell interaction with pathogen cells appear to be the main mechanisms of biocontrol of fire blight. Its ability to colonize, rapidly grow and survive in plant surface, and the fact that inhibited infections caused by *E. amylovora* in all the sensible organs, constitute interesting traits for an effective development under commercial conditions.

GENERAL DISCUSSION

Crop protection has been recently reoriented to a rational use of pesticides and to the reduction of the number of registered active ingredients more selective, less toxic and with a lower negative environmental impact (Gullino *et al*., 1994; Ragsdale and Sisler, 1994; Montesinos, 2003a). In the case of fire blight, the low effectiveness of the currently available methods for disease control, the development of resistance in some pathogens, the restrictions use of some products (e.g. streptomycin) in many countries and the widespread of fire blight across different areas, make necessary the development of additional control methods.

In the present study, a biological control methodology has been developed for treatment of fire blight, and for preventing dispersion of existing focus and introduction of fire blight in protected fruit production areas. The development of a biocontrol methodology needs the selection of biocontrol agents, formulation, production and delivery as a commercial product, and finally the implementation of the biocontrol in the plant protection management programs. For these purposes, the selection of a potential biocontrol agent and the characterization of its putative mechanisms of biocontrol of fire blight have constituted the main objectives of this PhD thesis.

The first objective proposed in the present study was the selection of representative strains of *E. amylovora* to be used in the screening of potential biocontrol agents of fire blight. A diverse collection of *E. amylovora* composed of 53 strains isolated from different sources (geographical location and plant hosts) was build up. Strains diversity was studied attending to cultural characteristics, detection by PCR and ELISA, metabolic profiles based on API and Biolog GN systems, intensity of infections in immature pear fruits and genetic diversity by RFLPs of *ams* gene and genomic DNA.

E. amylovora strains analyzed during the present work appeared very homogeneous according to the traits studied and these results agree with several studies (Paulin and Samson, 1973; Vantomme *et al.,* 1986; McManus and Jones, 1995; Vanneste, 1995; Beer *et al*., 1996; Kim *et al*., 1996; Momol *et al*., 1997). Nevertheless, some diversity was observed mainly in metabolic profiles, MRFLPs of genomic DNA and pathogenicity on immature pear fruits. In addition, variation on MRFLPs of genomic DNA was related to geographical distribution, finding that has already been described in other studies (Zhang *et al*., 1998; Jock *et al*., 2002). Whereas, variation on carbon source use profile and pathogenicity seem to be related to isolation plant host in accordance with the study of Kim *et al*. (1996) using the Biolog system. Our results agree with a certain host species-specificity described for some strains (Norelli *et al*., 1986; Beer *et al*., 1996).

A detailed study of virulence was performed based on the relationship between incidence of infections and concentration of pathogen cells (dose-disease curves) or time (disease progression curves).

Estimated parameters from dose-disease and disease progression curves permitted the classification of strains according to their "aggressiveness" (ED_{50}, I_{max}, r_g), "infectivity" (*MID*, t_0), or general virulence (*CVI*, *AUDPC*). Strains that presented a higher virulence were EPS101, UPN513, UPN544, USV1043, USV4512 and USV4576. All these strains were isolated in Spain from *Pyrus communis* except UPN544 that was isolated from *Crataegus* sp. In addition, highly virulent strains were grouped in group II described by API 20E system except EPS101, and in the group I for Biolog GN system except UPN513. These results indicate that virulence of strains through the immature pear test could be related in certain manner with the plant host and their metabolic profile. However, more extensive studies are required to get some conclusion.

Two strains of *E. amylovora* have been selected to be used in the screening for potential biocontrol agents of fire blight, the CUCM273 and EPS101. EPS101 was selected because it was isolated from our influence region and presented the common characteristics of most *E. amylovora* strains, reason why it was considered as a reference strain. CUCM273 was selected because it is probably the most divergent strain differing in serotype, did not present the typical white, domed, shiny, and mucoid colonies as typical strains.

Another objective was the optimization of model pathosystems to be applied in the selection of biocontrol agents of fire blight, and in interaction studies. Immature pear fruit, blossom and shoot infection assays were optimized using the deposition of a calibrated suspension of the pathogen on wounds produced in the leaves for the shoot assay or on the hypanthium surface of flowers in the blossom assay.

Once the pathogen strains were selected and model assay systems were set-up, potential biological control agents of fire blight were selected. Around 533 isolates, mainly of *P. fluorescens* and *E. herbicola* species, obtained from flowers, fruits and leaves of Rosaceous plants, were screened as potential biocontrol agents of fire blight disease using an immature pear fruit assay against strain CUCM273. At the end of the screening, sixty one isolates were selected because presented high efficacy in inhibition of infections caused by CUCM273. These antagonists were used for further studies in order to select the best suitable potential biocontrol agent of fire blight and to improve the knowledge on the characteristics involved in the biocontrol and the performance of screening methods. Although, only 10 out of the 61 strains retested against EPS101 maintained a high efficacy in the inhibition of *E. amylovora* infections in immature pear fruits. Therefore after the second trial 10 out of 533 were retained, corresponding to a 1.5% efficiency in the screening process. This efficiency is in accordance with studies on selection of biocontrol agents performed on other pathosystems using such as *Stemphylium vesicarium* in pear leaves (Montesinos et al., 1996) or *Sclerotinia sclerotiorum* in bean buds (Yuen *et al*., 1994) reporting efficacies of 7% and 3%, respectively.

Because the model of immature pear fruits is far from the natural conditions and some studies indicate that no correlation exist between results in fruits and flowers (Wilson *et al*., 1990; Pusey, 1992), more representative models such as flowers and shoots were included in the screening process. The blossom assay was used to confirm the potential of eight strains (EPS156e, EPS283e, EPS438, EPS460, EPS62e, EPS538, EPS684 and EPS734) as biocontrol agents of *E. amylovora*. After two trials, only the strain EPS62e of *P. fluorescens* was selected because it was the only that exhibited consistent high efficacy in pear blossoms. This strain also showed the ability to inhibit consistently infections produced by strain EPS101 of *E. amylovora* in wounded leaves obtaining the optimum control levels at 10^8 cfu·mL^{-1} when *E. amylovora* was applied at 10^7 cfu·mL^{-1}.

The sixty one isolates most effective against *E. amylovora* CUCM273 in immature pear fruits were characterized using the API system, *in vitro* antagonism on agar media against different plant pathogens, production of interesting compounds (antibiotics, siderophores, chitinases, indolacetic acid), level of inhibition of infections in immature pear fruits, hypersensitivity reaction and ice nucleation activity. Although, no relationship has been observed between the efficacy in the inhibition of infections caused by *E. amylovora* in immature pear fruits and API system profile, *in vitro* antagonism on agar media against different plant pathogens or production of antimetabolite compounds (antibiotics, siderophores, chitinases, indolacetic acid) according to correspondence analysis. These results contrast with the report of specific traits associated to biocontrol of *Pythium ultimum* such as the accumulation of C17:0 cyclopropane fatty acid and production of hydrogen cyanide by fluorescent pseudomonads (Ellis *et al*., 2000). A direct relationship between *in vitro* antagonism and inhibition of *E. amylovora* in immature pear fruits was not found. Only 23% of selected strains, pertaining to *P. fluorescens* and *E. herbicola* species, presented *in vitro* activity against *E. amylovora*, and most of the antagonists with high and consistent efficacy in immature pear fruits did not develop *in vitro* antagonism against *E. amylovora*. These results agree with other studies suggesting that antibiotic production is important in the biological control of *E. amylovora*, but additional mechanisms can be involved (Vanneste *et al*., 1992; Wilson *et al*., 1992; Nuclo *et al*., 1998).

At the end of the screening process, the strain EPS62e of *P. fluorescens* was selected. The strain was isolated from the surface of a pear fruit using a selective enrichment procedure and identified as *P. fluorescens.* It does not present undesirable characteristics like INA or induction of HR. This strain does not synthesize the most common antibiotics described for *P. fluorescens* (2,4-diacetylphloroglucinol, phenazine-1-carboxylic and pyrrolnitrin), nor produce compounds such as cyanide, indolacetic acid or chitinases. These results agree with the results reported for *P. fluorescens* A506 that does not exhibit antibiosis against *Erwinia amylovora in vitro* and hence it has been hypothesized that competition rather than antibiosis is involved in the mechanism of control (Wilson and Lindow, 1993). In contrast, most of biocontrol agents pertaining to *P. fluorescens* species described have shown the capacity to produce antibiotics. Some examples are the strain CHA0 that produces pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol and cyanide and control root diseases

(Duffy and Défago, 1999), and the EPS288 that produces cyanide and control fungal diseases in postharvest (Francés, 2000). The strain is tolerant to fungicides and insecticides, quite tolerant to copper hydroxide, kasugamycin and streptomycin, although it was sensible to wide-range action biocides like copper sulphate, copper oxychloride and fosetyl-aluminium. It presents a very limited spectrum of *in vitro* activity against bacterial plant pathogens and *E. amylovora* strains predominantly mediated by siderophores, but a broad range of activity in *ex vivo* assays against *E. amylovora* strains in immature pear fruits.

EPS62e presents a characteristic electrophoretic pattern of macrofragments obtained by digestion of the genomic DNA with the restriction endonuclease *Swa* I which differentiate it from other isolates, type strains and biocontrol agents of the same species. This RFLP pattern is a specific method for traceability studies required for patenting and registration of biocontrol agents (European Comission, 2001; Montesinos, 2003).

The last step in the development of a fire blight biological control agent was conducted to study the efficacy and consistency under controlled conditions. The study was focused in the determination of the potential of strain EPS62e in the control of fire blight on pear, the influence of antagonist and pathogen concentration on biocontrol efficacy, and the putative mechanisms of action.

As in other biocontrol agent-pathogen-host plant systems, the activity of EPS62e depends on the cell concentration of both the pathogen and the antagonist. Knowledge of antagonistic-pathogen density relationships provides data on the population levels of the antagonist required to achieve adequate disease control (Johnson, 1994, Montesinos and Bonaterra, 1996). In the present study several quantitative parameters have been determined from dose-response models describing the efficiency of the pathogen on disease development and of the biocontrol agent on disease control. The most useful were the median effective dose of the biocontrol agent and pathogen as well as the ratio between the median effective dose of the biocontrol agent and the pathogen (K_z/K_x). It was observed that all the parameters were influenced by the cultivar and plant organ. According to these parameters, the cultivars were arranged from more (Doyenne du Comice, Passe Crassane) to less susceptible (Blanquilla, Conference). Also, it was observed that flowers are more susceptible to fire blight infections than immature fruits though a lower efficiency was observed on immature pear fruits of Doyenne du Comice cultivar.

The median effective dose ratio was used to compare the efficiency of EPS62e among cultivars, plant materials and pathosystems. According to this parameter, the highest efficiency was obtained on immature pear fruits of Conference cultivar. In this plant material one cell of EPS62e was capable to inactivate twenty cells of *E. amylovora*. EPS62e was also highly effective in immature pear fruits of Passe Crassane and Blanquilla cultivars with ratios of 1 and 40, respectively. Similar results have been reported by Mandeel and Baker (1991) in the biocontrol of *Fusarium oxysporum* f. sp. *cucumerinum* on cucumber by *F. oxysporum* C14 with a ratio of 10, and in the case of the biocontrol of *Pythium* on tomato cultivars by *Bacillus cereus* UW85 with effective dose ratios between 1 and 5 cfu per oospore (Smith et

al., 1997). In contrast, EPS62e was less efficient in fruits and flowers of Doyenne du Comice cultivar, with a median effective dose ratio around 10^3, and in flowers of Conference cultivar, with values of 570. These values are similar to those observed in strain EPS5001 of *P. agglomerans* and in strains EPS288 and EPS381 of *P. fluorescens* against the fungus *Stemphylium vesicarium* on pear (Montesinos and Bonaterra, 1996). The results also agreed with those reported for strain EPS125 of *P. agglomerans* against *P. expansum* on apple (Moreno, 2003), and *Monilia laxa* and *Rhizopus stolonifer* on stone fruits (Bonaterra *et al.*, 2003).

Knowledge of the mechanism of action involved in the biocontrol process can permit the establishment of optimum conditions for implementing biological control in a given pathosystem (Cook, 1993; Handelsman and Stabb, 1996). Several mechanisms have been suggested to operate on biocontrol of fire blight, including antibiosis, induced resistance in the host and competition for space and limited sources between the biocontrol agetn and pathogen.

Interaction studies between EPS62e and *E. amylovora* were performed to determine the implication of antibiosis, induction of plant defence responses, and competition for space and nutrients.

The implication of antibiosis in the inhibition of *E. amylovora* was excluded because the strain does not produce antibiotics described for *P. fluorescens* neither inhibit in the *in vitro* agar inhibition test most of bacterial phytopathogens nor *E. amylovora* strains using different types of media. Moreover, it is unlikely that production of antibiotic substances is important because neither the cell-free culture filtrate of EPS62e amended with GA components nor physical separation by a membrane filter produce inhibition of pathogen growth. Only siderophore production has been related with the antagonism on plate against some *E. amylovora* strains indicating that siderophores could play a role on disease control.

Contrarily, in diluted pear juice or at high EPS62e:*E. amylovora* ratios it was observed a slight inhibition of growth of *E. amylovora* by EPS62e indicating that nutrient depletion is involved in inhibition. Some work was performed in order to study the potential of EPS62e to compete with *E. amylovora*, confirming its performance as good competitor of *E. amylovora* because it presents a greater maximum growth rate (μ_{max}), higher nutrient affinity (lower K_s) than *E. amylovora* on immature pear fruits juice, and a high *NOI* demonstrating that was capable to use most carbon sources used by *E. amylovora*.

Cell-to-cell interaction studies were also performed by means of the method described by Janisiewicz *et al.* (2000) based on the physical separation of cells through a semi-permeable membrane. Results confirmed that direct interaction is needed for inhibition of *E. amylovora* in culture broth. This is probably that occurs in immature pear fruits, flowers and whole plants, though other mechanisms may be involved like nutrients and space competition. In addition, studies performed at increasing concentrations and ratios of EPS62e and EPS101 using the same methodology allowed to estimate the rate of change (r) of relative growth of

E. amylovora. This parameter can be useful to compare antagonistic strains on the basis of their efficacy in the inhibition of *E. amylovora*.

EPS62e showed the capacity to delay fire blight infections on whole plants previously infiltrated with EPS62e suspensions. This is probably mediated by the induction of plant defense responses. However, an inconsistent effect has been observed and more exhaustive studies focused on the timing of application of EPS62e and analysis of the temporal expression of defense PR genes analyzed through reverse transcription polymerase chain reaction (RT-PCR) are required to prove the involvement of this mechanism on the biocontrol of fire blight by EPS62e. Several studies have been demonstrated that *Pseudomonas* spp. induce systemic resistance to a variety of above ground diseases, including wilt diseases, anthracnose, bacterial and viral diseases (Maurhofer *et al*., 1994; Liu *et al*., 1995; Wei *et al.*, 1996). Some examples are the biocontrol of soil-borne fungi and viruses in tomato by *Pseudomonas aeruginosa* strain 7NSK2, *P. fluorescens* strain CHA0 through the induction of systemic resistance mediated by salicylic acid (Siddiqui and Shaukat, 2004) or the inhibition of *P. syringae* pv *tomato* infections in *Arabidopsis* through induction of defense responses mediated by *P. fluorescens* WCS417r independently of salicylic acid accumulation (Pieterse *et al*., 1998). Although in all cases the induction is mediated by root colonization and Kloepper *et al*. (1992) introduced the term induced systemic resistance (ISR) to distinguish this phenomenon from the classical SAR (systemic acquired resistance).

Finally, EPS62e also showed the ability to colonize and survive well in wounds of immature apple and pear fruits and in the pear flower surface under controlled environment conditions, showing also the ability to inhibit fire blight infections in these plant organs. The strain showed the capacity to colonize and survive during ten days onto the flowers surface under field conditions at stable population levels around 10^6 cfu·corymb^{-1}. These results agree with reports of *E. herbicola* HL9N13 in hawthorn blossoms (Wilson *et al*., 1992), *E. herbicola* C9-1S in apple and pear blossoms (Johnson *et al*., 2000), and *P. fluorescens* A506 in pear flowers (Wilson and Lindow, 1993).

In conclusion, EPS62e is effective at moderately concentrations in preventive treatments for control of fire blight under controlled environment conditions, having the ability to colonize, rapidly grow and survive in plant surfaces and to perform preemptive exclusion of *E. amylovora* by surface colonization, nutrients depletion, and cell-to-cell antagonistic interaction.

CONCLUSIONS

1) The fifty three *E. amylovora* strains analyzed in the present work representing a wide range of origins were very homogeneous according to cultural characteristics, PCR and ELISA reaction, metabolic profiles based on API 20NE and Biolog GN systems and genetic diversity by RFLPs of *ams* gene and genomic DNA.

2) The study of the virulence of *E. amylovora* by means of dose-response relationships and disease progression kinetics showed significant differences among strains. Parameters estimated from curves allowed classification according to aggressiveness (ED_{50}, I_{max}, r_g), infectivity (*MID*, t_0), or general virulence (*CVI*, *AUDPC*). The most virulent strains were UPN513, USV2773, USV4512, USV4576, USV4501, USV1043, UPN544 and EPS101, while the less virulent were NCPPB311, UPN546, UPN609, UPN610 and EPS100.

3) Immature pear fruit, blossom and shoot assays were optimized for screening of biological control agents using inoculations based on the deposition of a calibrated suspension of the pathogen on wounds produced in the leaves and in immature pear fruits (shoot and fruit assay) or on the hypanthium surface of flowers in the blossom assay.

4) From the collection of *E. amylovora* strains, two were selected on the basis of virulence and cultural, nutritional, biochemical, and serological characteristics. Strain CUCM273 was selected because it was isolated in USA and it is probably the most divergent strain differing in serotype, and did not present the typical white, domed, shiny, mucoid colonies as typical strains; and strain EPS101 because it was isolated from our influence region in Lleida and presented the common characteristics of most *E. amylovora* strains.

5) Ten out of 533 epiphytic bacterial isolates from different plant parts, host and species developed high efficacy and consistency of results of inhibition of infections caused by *E. amylovora* CUCM273 and EPS101 in immature pear fruits. Finally, only strain EPS62e of *P. fluorescens* was selected after several tests on immature fruits, flowers and young shoots of pear.

6) No relationships were observed between efficacy in the inhibition of infections caused by *E. amylovora* in immature pear fruits and API system profile, *in vitro* antagonism on agar media against different plant pathogens or production of antimetabolite compounds (antibiotics, siderophores, chitinases, indolacetic acid) according to correspondence analysis. Therefore, a pattern of common characteristics among the

antagonists with high efficacy against *E. amylovora* on immature pear fruits was not found.

7) Strain EPS62e was identified as *P. fluorescens,* does not produce known antibiotics nor other compounds like cyanide, indolacetic acid, and chitinases, neither it presents ice nucleation activity nor induce HR on tobacco. The strain is tolerant to fungicides and insecticides, quite tolerant to kasugamycin and streptomycin, although it is sensible to wide-range action biocides like copper sulphate, copper oxychloride and fosetyl-aluminium. It presents a very limited spectrum of *in vitro* activity against bacterial plant pathogens and *E. amylovora* strains predominantly mediated by siderophores, but a broad range of activity in *ex vivo* assays against *E. amylovora* strains on immature pear fruits. Strain EPS62e exhibits a characteristic electrophoretic pattern of macrofragments of digestion of genomic DNA with *Swa* I. This is a differential characteristic from other isolates, type strains and biocontrol agents of the same species.

8) The efficacy of EPS62e depends on the concentration of the biocontrol agent and pathogen, the host plant and plant material. The median effective dose (K_z) ranges from $9.5 \cdot 10^2$ in immature fruits of cultivar Conference to $9.2 \cdot 10^6$ cfu·mL^{-1} in immature fruits of cultivar Doyenne du Comice. The median effective dose ratio was used to compare the efficiency of EPS62e among cultivars and plant materials. The highest efficiency in the inhibition of *E. amylovora* infections was obtained on immature pear fruits of Conference cultivar where one cell of EPS62e was capable to counteract twenty cells of *E. amylovora*. EPS62e was also highly effective in immature pear fruits of Passe Crassane and Blanquilla cultivars with ratios of 1:1 and 40:1, respectively. In contrast, EPS62e was less efficient in fruits and flowers of Doyenne du Comice cultivar, with a median effective dose ratio around 10^3, and in flowers of Conference cultivar, with values of 570.

9) The implication of antibiosis in the inhibition of *E. amylovora* by EPS62e was excluded because neither the cell-free spent GA medium of EPS62e amended with nutrients nor physical separation by a membrane filter produced inhibition of the growth of *E. amylovora*. In addition, EPS62e does not produce the most common antibiotics described on *P. fluorescens* neither inhibits most of bacterial phytopathogens nor of *E. amylovora* strains on agar plate culture using different media.

10) Direct interaction was involved in the inhibition of *E. amylovora* by EPS62e because cell-to-cell contact was needed. The effect of direct interaction was influenced by the EPS62e/EPS101 ratio. The rate of change of growth rate (r) of *E. amylovora* with the ratio was found useful to compare the efficacy of antagonistic strains.

11) EPS62e exhibits a good nutrient competition ability because it presents a greater maximum growth rate (μ_{max}), higher nutrient affinity (lower K_s) than *E. amylovora* on immature pear fruits juice, and a high *NOI* demonstrating that was capable of use most carbon sources used by *E. amylovora*.

12) EPS62e showed the capacity to delay fire blight infections in pear plants previously infiltrated with a EPS62e suspension. However, an inconsistent effect of the induction of plant defense responses was observed and more exhaustive studies focused on the timing of application of EPS62e are required to prove the involvement of this mechanism in fire blight control.

13) EPS62e showed the ability to colonize and survive well in wounds of immature apple and pear fruits and in the pear flower surface under controlled environment conditions. The strain also showed the capacity to colonize and survive almost during ten days onto the flowers surface under field conditions at stable population levels around 10^6 cfu·corymb^{-1}.

14) It is finally concluded that all above mentioned traits constitute interesting properties for an effective development of strain EPS62e as a fire blight biological control agent under commercial conditions.

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ANNEXES

***ANNEX 1*. Buffers**

Antioxidant maceration buffer (Gorris *et al.*, 1996)

Polyvinilpyrrolidone (PVP-10)	20 g
Mannitol	10 g
Ascorbic acid	1.76 g
Reduced glutathion	3 g
PBS	10 mM [pH 7.2]
Distilled water	1 L
Adjust [pH to 7]	
Sterilized by filtration	

Carbonate buffer [pH 9.6]

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled water	1 L
Adjust pH to 9.6	

Physiological buffered water (AFT) [pH 7.2-7.4]

NaCl	8 g
$NaH_2PO_4 \cdot 2H_2O$	0.4 g
$Na_2HPO_4 \cdot 12H_2O$	2.7 g
Distilled water	1 L
Adjust pH to 7.2-7.4	

DNA extraction buffer [pH 7.5] (Llop *et al.*, 1999)

Tris Base [pH 7.5]	24.2 g
NaCl	14.6 g
EDTA	9.3 g
Sodium Dodecyl Sulfate (SDS)	5 g
Polyvinilpyrrolidone PVP-10	20 g
Distilled water	1 L
Sterilized by filtration	

Tris-Acetate-EDTA (TAE) buffer 50X [pH 8.0]

Tris base	242 g
Glacial acetic acid	57.1 mL
EDTA (0.5M) [pH 8.0]	100 mL
Distilled water	Adjust volume to 1 L

PET IV buffer [pH 7.6]

Tris base (1 M)	10 mL
NaCl (5 M)	200 mL
Distilled water	Adjust volume to 1 L

Adjust pH to 7.6

20 min sterilization by autoclaving (121 ºC)

Tris-EDTA (TE) [pH 8.0]

Tris base (1 M)	10 mL
EDTA (0.5M) [pH 8.0]	0.2 mL
Distilled water	Adjust volume to 1 L

Adjust pH to 8.0

20 min sterilization by autoclaving (121 ºC)

Digestion buffer [pH 9.5]

proteinase K	0.5 mg per mL
$CaCl_2$	1 mM
lauroylsarcosine	1%
glycine	10 mM
EDTA	0.25 M

HEPES buffer 10X [pH 8.0]

HEPES	9.532 g
Sodium acetate	5.444 g
EDTA	1.488 g

Adjust pH to 8.0

20 min sterilization by autoclaving (121 ºC)

Buffered peptone water [pH 7.2-7.4]

Na_2HPO_4	7.10 g
KH_2PO_4	2.72 g
Peptone	1g

Adjust pH to 7.2-7.4

20 min sterilization by autoclaving (121 ºC)

Substrate buffer [pH 9.8]

Dietanolamina	97 ml
NaN_3 (optional)	0.2 g

Adjust pH to 9.8

Adjust volume to 1 L

20 min sterilization by autoclaving (121 ºC)

ANNEX 2. Media and reagents

King's B medium (KB) (King *et al.*, 1954)

Proteose peptone	20 g
Glycerol	10 mL
K_2HPO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	1.5 g
Agar	15 g
Distilled water	1 L

Adjust pH to 7-7.2

20 min sterilization by autoclaving (121 ºC)

Sucrose nutrient agar medium 5% (SNA)

Yeast extract	2 g
Bactopeptone	5 g
NaCl	5 g
Sucrose	50 g
Agar	15 g
Distilled water	1 L

Adjust pH to 7-7.2

20 min sterilization by autoclaving (121 ºC)

CCT medium (Ishimaru and Klos, 1984)

Part 1:

Sucrose	100 g
Sorbitol	10 g
Niaproof	1.2 ml
Crystal violet	2 ml(sol. 0.1 % ethanol)
Nutrient agar	23 g
Distilled water	1 L

Adjust pH to 7.0-7.2

10 min sterilization by autoclaving (121 ºC)

Part 2 (to add to part 1 after sterilisation):

Thallium nitrate	2 ml (1%w/v aqueous solution)
Cycloheximide	0,05 g

Sterilise by filtration (0.45 µ)

Add to 1 L of the sterile first part (at about 45 ºC)

Miller-Schroth agar medium (MS) (Miller and Schroth, 1972)

Manitol	10 g
L-asparagine	3 g
K2HPO4	2 g
Sodium taurocolate	2.5 g
MgSO4·7H2O	2 g
Nicotinic acid	0.5 g
Anionic tergitol	0.1 mL
Nitrilotriacetic acid (5%)	10 mL
Bromothymol blue (1%)	9 mL
Neutral red (0.5%)	2.5 mL
Cobalt chloride (1.65%)	10 mL
Agar	15 g
Distilled water	1 L

Adjust pH to 7-7.2

20 min sterilization by autoclaving (121 ºC)

Luria Bertani agar medium (LB) (Maniatis *et al*., 1982)

Triptone	10 g
NaCl	10 g
Yeast extract	5 g
Agar	15 g
Distilled water	1 L

Adjust pH to 7.0-7.2

20 min sterilization by autoclaving (121 ºC)

Potato dextrose agar (PDA)

Potato-dextrose ager (DIFCO 0013-17-6)

Adjust pH to 7.0-7.2

20 min sterilization by autoclaving (121 ºC)

Chitinase test medium (Frändberg and Scnürer, 1998)

Colloidal chitin*	1.5 g
K_2HPO_4	2.7 g
KH_2PO_4	0.3 g
$MgSO_4 \cdot 7H_20$	0.7 g
NaCl	0.5 g
kCl	0.5 g
Yeast extract	0.13 g
Agar	20 g
Distilled water	1 L

Adjust pH to 7.0-7.2

20 min sterilization by autoclaving (121 ºC)

* Colloidal chitin was prepared as described by Rodriguez-Kabana *et al*. (1983).

Glucose asparagine (GA)

Glucose	20 g
L-asparagine	0.3 g
Nicotinic acid	0.05 g
K_2HPO_4	11.5 g
KH_2PO_4	4.5 g
$MgSO_4 \cdot 7H_20$	0.12 g
Distilled water	1 L
Agar	15 g

Adjust pH to 7.0-7.2

20 min sterilization by autoclaving (121 ºC)

Trypticase soy broth agar (TSBA)

Triptone soy agar	40 g
Distilled water	1 L

Adjust pH to 7.0-7.2

20 min sterilization by autoclaving (121 ºC)

Salkowski reagent

FeCl3 (2%)	0.5 M
$HClO_4$	35%

Picrate reagent

Picric acid	0.5%
$NaCO_3$	2%

Biolog universal growth agar (BUG)

BUGTM agar	57 g
Distilled water	1 L

Adjust pH to 7.3 ± 0.1

20 min sterilization by autoclaving (121 ºC)

CYE agar

Casitone	1.7 g
Yeast extract	0.37 g
Glycerol	2 g
Agar	15 g
Distilled water	1 L

Adjust pH to 7.0-7.2

20 min sterilization by autoclaving (121 ºC)

ANNEX 3

Estimated parameters and goodness-of-fit of the modified Gompertz model for disease progression curves of 53 *E. amylovora* strains inoculated at two concentrations

		10^3 cfu·mL^{-1}					10^5 cfu·mL^{-1}				
Strain	**Repetition**	B_g	r_g	t_0	**R²**	**P>F**	B_g	r_g	t_0	**R²**	**P>F**
USV1043	1	1.99	0.56	3.67	0.98	0.0000	15.68	1.69	1.58	0.99	0.0000
	2	1.92	0.73	4.81	0.97	0.0000	12.41	1.64	2.11	0.99	0.0000
	3	2.79	1.69	6.56	0.99	0.0000	13.01	1.64	2.08	0.99	0.0000
USV1000	1	5.03	0.87	2.85	0.98	0.0000	8.21	1.05	2.53	0.99	0.0000
	2	6.99	0.85	3.10	0.98	0.0000	7.01	0.87	2.53	0.99	0.0000
	3	3.34	0.69	3.58	0.97	0.0000	9.14	1.43	2.67	0.99	0.0000
EPS101	1	6.21	0.90	2.94	0.99	0.0000	8.12	1.43	1.54	0.99	0.0000
	2	3.94	0.85	3.65	0.98	0.0000	11.34	1.46	1.83	0.99	0.0000
	3	3.44	0.76	3.95	0.99	0.0000	6.53	1.49	2.40	0.98	0.0000
USV2194	1	0.55	0.96	8.59	0.93	0.0000	1.72	0.36	5.06	0.84	0.0005
	2	0.69	1.03	8.99	0.98	0.0000	1.74	0.39	5.20	0.88	0.0002
	3	-	-	-	-	-	1.44	0.56	5.76	0.94	0.0000
NCPPB3159	1	11.46	1.38	4.75	0.99	0.0000	13.94	1.20	3.69	0.97	0.0000
	2	9.34	1.18	3.65	0.99	0.0000	10.32	0.94	2.56	0.98	0.0000
	3	10.80	1.38	4.78	0.99	0.0000	13.94	1.20	3.38	0.94	0.0000
NCPPB3548	1	0	0	0	-	-	5.96	1.01	4.12	0.99	0.0000
	2	0	0	0	-	-	5.35	1.01	4.67	0.98	0.0000
	3	0	0	0	-	-	4.51	0.97	5.20	0.92	0.0000
NCPPB1734	1	4.64	1.06	3.78	0.99	0.0000	8.79	0.80	2.28	0.99	0.0000
	2	3.63	0.99	4.39	0.99	0.0000	11.13	0.95	1.94	0.99	0.0000
	3	3.49	0.79	4.42	0.99	0.0000	7.18	0.79	2.88	0.98	0.0000
NCPPB1819	1	3.30	0.78	5.46	0.98	0.0000	9.13	0.84	3.76	0.98	0.0000
	2	3.73	0.74	5.45	0.99	0.0000	4.46	0.67	3.92	0.99	0.0000
	3	3.91	0.91	5.52	0.98	0.0000	6.12	0.93	5.52	0.99	0.0000
NCPP2080	1	3.63	0.86	5.08	0.99	0.0000	6.09	1.20	3.02	0.99	0.0000
	2	3.02	0.84	5.42	0.99	0.0000	5.59	1.20	3.09	0.99	0.0000
	3	3.38	0.86	5.36	0.99	0.0000	3.61	0.77	2.97	0.98	0.0000
NCPPB2291	1	0	0	0	-	-	0	0	0	-	-
	2	0	0	0	-	-	0	0	0	-	-
	3	0	0	0	-	-	0	0	0	-	-
EAZ4	1	0	0	0	-	-	3.13	0.75	4.91	0.97	0.0000
	2	0	0	0	-	-	3.98	1.34	6.24	0.98	0.0000
	3	0	0	0	-	-	2.94	1.19	6.47	0.97	0.0000
EAZ7	1	0	0	0	-	-	2.79	0.90	4.82	0.99	0.0000
	2	0	0	0	-	-	3.06	0.67	3.82	0.96	0.0000
	3	0	0	0	-	-	4.84	0.85	3.88	0.97	0.0000
EAZ9	1	0	0	0	-	-	6.92	0.75	4.75	0.98	0.0000
	2	0	0	0	-	-	7.55	1.15	4.47	0.99	0.0000
	3	0	0	0	-	-	9.68	1.39	4.66	0.99	0.0000

(Continue)

		10^3 cfu·mL^{-1}					10^5 cfu·mL^{-1}				
Strain	**Repetition**	B_g	r_g	t_0	**R²**	**P>F**	B_g	r_g	t_0	**R²**	**P>F**
EAZ13	1	0	0	0	-	-	4.68	0.82	4.96	0.97	0.0000
	2	0	0	0	-	-	5.12	0.74	4.18	0.99	0.0000
	3	0	0	0	-	-	4.52	0.72	5.03	0.98	0.0000
CUCM273	1	5.18	1.03	3.35	0.98	0.0000	7.02	1.13	1.96	0.99	0.0000
	2	4.96	1.46	4.37	0.99	0.0000	4.76	1.08	1.98	0.99	0.0000
	3	3.04	0.79	4.59	0.97	0.0000	9.98	1.18	1.95	0.98	0.0000
NCPPB311	1	0	0	0	-	-	0	0	0	-	-
	2	0	0	0	-	-	0	0	0	-	-
	3	0	0	0	-	-	0	0	0	-	-
NCPPB2291	1	0	0	0	-	-	0	0	0	-	-
	2	0	0	0	-	-	0	0	0	-	-
	3	0	0	0	-	-	0	0	0	-	-
NCPPB595	1	3.99	0.50	7.85	0.98	0.0000	4.91	0.89	5.33	0.94	0.0000
	2	3.99	0.50	7.85	0.99	0.0000	4.78	1.33	6.47	0.98	0.0000
	3	4.09	0.77	8.16	0.99	0.0000	4.59	0.94	6.24	0.99	0.0000
NCPPB683	1	5.03	0.88	3.98	0.96	0.0000	5.81	0.90	3.01	0.98	0.0000
	2	5.48	0.84	3.53	0.97	0.0000	5.77	0.99	2.93	0.99	0.0000
	3	4.46	0.85	3.50	0.98	0.0000	4.17	0.76	3.47	0.97	0.0000
EAZ1	1	6.21	0.90	2.94	0.98	0.0000	7.38	0.75	1.46	0.98	0.0000
	2	3.51	1.34	4.71	0.99	0.0000	6.21	0.94	1.71	0.98	0.0000
	3	3.17	1.14	4.88	0.98	0.0000	6.97	0.79	2.04	0.97	0.0000
NCPPB2791	1	0	0	0	-	-	11.46	1.65	5.75	0.98	0.0000
	2	0	0	0	-	-	7.99	2.36	6.25	0.99	0.0000
	3	0	0	0	-	-	7.98	1.19	4.64	0.98	0.0000
UPN524	1	0	0	0	-	-	5.21	0.77	2.62	0.98	0.0000
	2	0	0	0	-	-	6.20	0.90	2.94	0.98	0.0000
	3	0	0	0	-	-	4.47	0.97	3.89	0.99	0.0000
UPN529	1	4.99	0.53	3.86	0.98	0.0000	10.67	1.31	2.37	0.99	0.0000
	2	5.16	0.48	3.71	0.99	0.0000	12.23	1.79	2.98	0.98	0.0000
	3	6.75	1.13	5.50	0.99	0.0000	10.87	1.44	2.74	0.98	0.0000
UPN530	1	9.79	1.46	3.93	0.99	0.0000	10.31	0.94	2.56	0.97	0.0000
	2	11.12	2.09	4.81	0.98	0.0000	10.01	1.08	3.30	0.99	0.0000
	3	11.37	1.68	4.75	0.98	0.0000	9.94	0.95	3.06	0.98	0.0000
UPN536	1	7.96	1.19	3.64	0.97	0.0000	10.63	1.07	1.97	0.97	0.0000
	2	6.60	0.95	3.48	0.98	0.0000	10.80	1.98	3.78	0.99	0.0000
	3	5.76	1.03	4.25	0.97	0.0000	13.68	1.39	3.41	0.99	0.0000
	1	6.21	0.90	2.94	0.98	0.0000	11.46	1.87	2.75	0.99	0.0000
	2	6.02	0.97	3.59	0.99	0.0000	11.36	1.18	2.76	0.99	0.0000
	3	4.84	0.85	3.64	0.99	0.0000	14.64	1.91	3.00	0.99	0.0000
UPN544	1	9.64	0.75	2.94	0.98	0.0000	11.99	1.39	1.50	0.99	0.0000
	2	7.45	0.79	4.18	0.98	0.0000	9.88	1.24	1.56	0.97	0.0000
	3	6.72	0.86	4.72	0.99	0.0000	11.47	1.98	2.75	0.99	0.0000

(Continue)

		10^3 cfu·mL^{-1}					10^5 cfu·mL^{-1}				
Strain	**Repetition**	B_g	r_g	t_0	**R²**	**P>F**	B_g	r_g	t_0	**R²**	**P>F**
UPN546	1	0	0	0	-	-	2.69	0.18	7.8	0.79	0.0013
	2	0	0	0	-	-	3.42	0.28	7.97	0.79	0.0014
	3	0	0	0	-	-	-	-	-	-	-
UPN562	1	0	0	0	-	-	2.97	0.48	7.32	0.97	0.0000
	2	0	0	0	-	-	2.05	0.36	7.73	0.92	0.0001
	3	0	0	0	-	-	1.60	0.34	7.86	0.82	0.0007
UPN575	1	0	0	0	-	-	4.11	0.95	3.98	0.99	0.0000
	2	0	0	0	-	-	3.46	0.76	4.98	0.95	0.0000
	3	0	0	0	-	-	2.92	0.53	5.76	0.97	0.0000
UPN576	1	3.87	0.54	3.96	0.99	0.0000	4.21	0.67	3.21	0.95	0.0000
	2	3.04	0.59	3.98	0.98	0.0000	5.42	1.46	5.34	0.99	0.0000
	3	3.91	0.49	4.52	0.99	0.0000	6.13	1.34	5.29	0.98	0.0000
EPS100	1	2.82	0.28	6.16	0.95	0.0000	2.76	0.27	5.56	0.89	0.0001
	2	3.16	0.13	5.92	0.76	0.0027	3.52	0.23	5.47	0.92	0.0000
	3	3.16	0.13	5.92	0.76	0.0027	3.43	0.21	5.83	0.89	0.0001
UPN588	1	2.19	0.32	7.53	0.94	0.0000	7.03	1.69	5.05	0.99	0.0000
	2	3.05	0.30	7.42	0.94	0.0000	5.26	1.39	5.09	0.98	0.0000
	3	2.22	0.23	7.49	0.92	0.0000	5.69	1.20	5.07	0.99	0.0000
USV2773	1	5.28	1.46	4.35	0.99	0.0000	11.19	1.69	2.78	0.99	0.0000
	2	6.03	1.46	4.26	0.99	0.0000	11.19	1.69	2.78	0.99	0.0000
	3	5.03	1.34	4.44	0.99	0.0000	14.17	1.91	3.02	0.99	0.0000
USV4300	1	8.27	1.92	4.25	0.99	0.0000	9.00	1.34	3.01	0.99	0.0000
	2	9.61	1.43	3.63	0.99	0.0000	13.02	1.69	3.08	0.99	0.0000
	3	11.03	1.94	4.12	0.99	0.0000	13.83	1.85	3.09	0.99	0.0000
USV4320	1	7.27	2.05	4.40	0.99	0.0000	8.45	1.34	3.06	0.99	0.0000
	2	6.34	1.69	4.11	0.99	0.0000	13.03	1.64	3.08	0.99	0.0000
	3	7.37	1.46	4.12	0.99	0.0000	13.03	1.64	3.08	0.99	0.0000
USV4408	1	7.88	1.98	3.95	0.99	0.0000	13.01	1.64	1.94	0.99	0.0000
	2	8.77	1.92	4.22	0.99	0.0000	13.01	1.64	3.08	0.99	0.0000
	3	10.76	1.80	4.06	0.99	0.0000	9.98	1.18	2.09	0.99	0.0000
UPN500	1	5.03	1.34	4.44	0.99	0.0000	11.81	1.79	3.01	0.99	0.0000
	2	5.30	1.43	4.04	0.99	0.0000	11.81	1.79	3.01	0.99	0.0000
	3	5.42	1.80	4.44	0.99	0.0000	11.63	1.20	2.49	0.99	0.0000
Ea115.2	1	0	0	0	-	-	7.73	0.70	2.55	0.96	0.0000
	2	0	0	0	-	-	7.80	0.62	2.57	0.97	0.0000
	3	0	0	0	-	-	6.30	0.56	1.75	0.97	0.0000
UPN506	1	0	0	0	-	-	10.21	1.75	3.04	0.97	0.0006
	2	0	0	0	-	-	8.26	1.05	2.53	0.98	0.0001
	3	0	0	0	-	-	14.16	1.91	3.02	0.99	0.0000
IVIA1614.2	1	4.69	0.72	2.87	0.98	0.0000	9.34	0.85	1.87	0.99	0.0000
	2	4.58	1.13	3.33	0.99	0.0000	9.67	1.23	2.17	0.99	0.0000
	3	4.92	0.87	2.94	0.98	0.0000	11.16	1.01	1.95	0.99	0.0000

(Continue)

		10^3 cfu·mL^{-1}					10^5 cfu·mL^{-1}				
Strain	**Repetition**	B_g	r_g	t_0	**R²**	**P>F**	B_g	r_g	t_0	**R²**	**P>F**
OMP-BO1185	1	2.54	0.90	3.35	0.99	0.0000	3.75	0.68	1.57	0.94	0.0000
	2	2.63	0.88	3.49	0.98	0.0000	6.01	1.05	1.58	0.99	0.0000
	3	3.53	0.77	3.17	0.97	0.0000	4.04	0.77	1.48	0.98	0.0000
UPN513	1	7.00	1.34	3.20	0.98	0.0000	13.91	1.43	1.16	0.99	0.0000
	2	6.02	1.19	3.14	0.99	0.0000	11.19	1.70	1.78	0.98	0.0000
	3	10.12	1.64	3.24	0.99	0.0000	11.13	2.04	1.81	0.98	0.0000
UPN514	1	7.70	1.23	2.36	0.99	0.0000	8.19	1.02	1.81	0.99	0.0000
	2	5.26	0.83	2.15	0.97	0.0000	11.19	1.70	2.78	0.99	0.0000
	3	11.13	2.04	2.81	0.99	0.0000	8.76	1.34	2.03	0.98	0.0000
USV4512	1	4.19	0.90	2.75	0.99	0.0000	9.66	1.46	1.94	0.98	0.0000
	2	6.32	1.22	2.32	0.99	0.0000	9.89	1.43	1.61	0.99	0.0000
	3	7.03	1.38	2.99	0.99	0.0000	11.19	1.70	1.78	0.99	0.0000
USV4576	1	9.69	1.46	2.94	0.99	0.0000	12.90	1.13	1.42	0.99	0.0000
	2	10.12	1.64	3.24	0.99	0.0000	11.19	1.70	1.78	0.99	0.0000
	3	10.39	1.43	2.58	0.99	0.0000	14.17	1.85	2.02	0.99	0.0000
CFBP1430	1	9.85	1.43	2.62	0.99	0.0000	11.57	0.94	0.86	0.99	0.0000
	2	11.19	1.70	2.78	0.99	0.0000	11.84	1.08	1.14	0.99	0.0000
	3	9.37	1.19	2.42	0.99	0.0000	10.02	1.14	1.87	0.99	0.0000
PMV6076	1	0	0	0	-	-	0	0	0	-	-
	2	0	0	0	-	-	0	0	0	-	-
	3	0	0	0	-	-	0	0	0	-	-
UPN609	1	0	0	0	-	-	2.92	0.42	7.34	0.99	0.0003
	2	0	0	0	-	-	*				
	3	0	0	0	-	-	*				
UPN610	1	0	0	0	-	-	2.93	0.44	7.36	0.99	0.0003
	2	0	0	0	-	-	*				
	3	0	0	0	-	-	*				
UPN611	1	8.77	1.34	3.03	0.99	0.0000	11.01	1.18	1.86	0.98	0.0000
	2	7.95	1.18	3.14	0.99	0.0000	15.23	1.31	2.10	0.99	0.0000
	3	7.89	1.23	3.34	0.99	0.0000	11.84	1.19	2.23	0.99	0.0000
EAZ3	1	13.03	1.64	3.08	0.99	0.0000	19.08	1.68	2.45	0.98	0.0000
	2	12.49	1.80	3.40	0.99	0.0000	13.36	1.39	2.43	0.99	0.0000
	3	12.49	1.80	3.40	0.99	0.0000	13.36	1.39	2.43	0.99	0.0000
USV4499	1	4.65	1.2	3.55	0.99	0.0000	14.73	1.56	3.05	0.98	0.0000
	2	7.71	1.23	3.35	0.99	0.0000	11.04	1.20	2.63	0.99	0.0000
	3	5.88	1.04	3.33	0.99	0.0000	11.34	1.46	2.83	0.99	0.0000
USV4500	1	5.62	0.72	3.59	0.99	0.0000	7.16	1.03	2.04	0.98	0.0000
	2	4.16	0.84	4.80	0.99	0.0000	7.67	1.18	2.17	0.99	0.0000
	3	3.67	0.66	4.74	0.99	0.0000	7.67	1.18	2.17	0.99	0.0000
USV4496	1	4.12	0.61	5.13	0.99	0.0000	11.19	1.69	2.78	0.99	0.0000
	2	3.12	0.41	5.72	0.95	0.0000	11.59	1.39	2.53	0.99	0.0000
	3	3.57	0.48	6.38	0.99	0.0000	13.01	1.64	3.08	0.99	0.0000

* Model could not be fitted for this experimental data.

ANNEX 4

List of samples according to species, rootstock, processed plant part and reference of isolates obtained

Sample	Species	Rootstock	Plant part	Isolates obtained
1	*Prunus insitia*	CIRPAC-2	aerial	650-651
2	*P. insitia*	CIRPAC-2	radicular	652-653-654-655
3	*P.persica x P. davidiana*	BARRIER	aerial	657-658-659
4	*P. persica x P. davidiana*	BARRIER	radicular	660-661-662-663-814
5	*P. avium*	SANTA LUCIA 64	aerial	664-665-666
6	*P. avium*	SANTA LUCIA 64	radicular	667-668-669-817
7	*P. besseyi x P. salicina*	DEEP PURPLE	aerial	670-671-672-673
8	*P. besseyi x P. salicina*	DEEP PURPLE	radicular	674-675-676-677
10	*P. dulcis x P. persica*	FELINEM	radicular	679-680-681-718
11	*P. domestica x P. persica*	MICROBAC2	aerial	682-683-684-685-686
12	*P. domestica x P. persica*	MICROBAC2	radicular	687-688-689-690
13	*P. domestica*	MARIANO 2624	aerial	691-692-693-694
14	*P. domestica*	MARIANO 2624	radicular	695-696-697-698
15	*P. persica x P. davidiana*	MAYOR	aerial	699-700-701-702
16	*P. persica x P. davidiana*	MAYOR	radicular	703-704-705-706
17	*P. persica x P. davidiana*	CADAMAN	aerial	707-708-709-710-711-712
18	*P. persica x P. davidiana*	CADAMAN	radicular	713-714-715-717
19	*P. persica x P. amygdalus*	GF-677	aerial	719-720-721
20	*P. persica x P. amygdalus*	GF-677	radicular	722-723-724-725-726
21	*P. persica x P. dulcis*	REBATO	aerial	727-728-729-730
22	*P. persica x P. dulcis*	REBATO	radicular	731-732-733-734-807-808-809
23	*P. persica x P. dulcis*	ADAFUEL	aerial	735-737-738
24	*P. persica x P. dulcis*	ADAFUEL	radicular	739-740-741-742-743
25	*P. persica x P. dulcis*	MONEGRO	aerial	744-745-746-747
26	*P. persica x P. dulcis*	MONEGRO	radicular	736-748-749-750-751-760
27	*P. cerasifera x P. munsioniana*	MARIANA 4001	aerial	752-753-754-755
28	*P. cerasifera x P. munsioniana*	MARIANA 4001	radicular	756-757-758
29	*P. insititia*	ADESOTO	aerial	759-761-762
30	*P. insititia*	ADESOTO	radicular	763-764-765-766-767
31	*P. domestica*	TETRA	aerial	768-769-770-771-810
32	*P. domestica*	TETRA	radicular	772-773-774-775-776-811-815
33	*P. domestica*	29C MYROBLAN	radicular	777-778-779-780-782-812
34	*P. domestica*	TETRA	substrat	783-784-786-787-788-789
35	*P. persica x P. amygdalus*	GF'	aerial	790-791-792-793
36	*P. persica x P. amygdalus*	GF'	radicular	794-795-796-797
37	*P. cerasifera x P. munsioniana*	MARIANA 2624	aerial	678-798-799-800-801
38	*P. persica x P. belsiana x P. domestica*	ISHTARA	aerial	716-802-803-804-805-816-818
39	*P. persica x P. dulcis*	LUCERO	radicular	781-785-805-806-813

ANNEX 5

List of isolates according to host specie, source organ, fluorescence production on KB agar, colony colour on LB agar and specie referred of 169 epiphytic isolates obtained from plant samples

Isolate	Plant species	Organ	Fl[x]	Colony[y]	Species referred[z]
650	*Prunus insitia*	leaf	-	white	*nd*
651	*Prunus insitia*	root	-	orange	*E. herbicola*
652	*Prunus insitia*	root	-	white	*nd*
653	*Prunus insitia*	root	+	white	*P. fluorescens*
654	*Prunus insitia*	root	+	white	*P. fluorescens*
655	*Prunus insitia*	root	+	white	*nd*
656	*P. persica x P. davidiana*	leaf	-	orange	*E. herbicola*
657	*P. persica x P. davidiana*	leaf	+	white	*P. fluorescens*
658	*P. persica x P. davidiana*	leaf	-	white	*nd*
659	*P. persica x P. davidiana*	leaf	-	white	*nd*
660	*P. persica x P. davidiana*	root	-	orange	*E. herbicola*
661	*P. persica x P. davidiana*	root	+	white	*P. fluorescens*
662	*P. persica x P. davidiana*	root	+	white	*P. fluorescens*
663	*P. persica x P. davidiana*	root	+	white	*P. fluorescens*
664	*P. avium*	leaf	+	white	*P. fluorescens*
665	*P. avium*	leaf	+	white	*P. fluorescens*
666	*P. avium*	leaf	-	white	*nd*
667	*P. avium*	root	-	orange	*E. herbicola*
668	*P. avium*	root	+	white	*P. fluorescens*
669	*P. avium*	root	-	orange	*E. herbicola*
670	*P. besseyi x P. salicina*	leaf	+	white	*P. fluorescens*
671	*P. besseyi x P. salicina*	leaf	+	white	*P. fluorescens*
672	*P. besseyi x P. salicina*	leaf	-	orange	*E. herbicola*
673	*P. besseyi x P. salicina*	leaf	-	white	*nd*
674	*P. besseyi x P. salicina*	root	+	white	*P. fluorescens*
675	*P. besseyi x P. salicina*	root	+	white	*P. fluorescens*
676	*P. besseyi x P. salicina*	root	+	white	*P. fluorescens*
677	*P. besseyi x P. salicina*	root	+	white	*P. fluorescens*
678	*P. domestica*	leaf	-	orange	*E. herbicola*
679	*P. dulcis x P. persica*	root		white	*nd*
680	*P. dulcis x P. persica*	root	+	white	*P. fluorescens*
681	*P. dulcis x P. persica*	root	+	white	*P. fluorescens*
682	*P. comestica x P. persica*	leaf	-	orange	*E. herbicola*
683	*P. comestica x P. persica*	leaf	-	orange	*E. herbicola*
684	*P. comestica x P. persica*	leaf	+	white	*P. fluorescens*
685	*P. comestica x P. persica*	leaf	-	white	*nd*
686	*P. comestica x P. persica*	leaf	-	orange	*E. herbicola*
687	*P. comestica x P. persica*	root	+	white	*P. fluorescens*
688	*P. comestica x P. persica*	root	-	white	*nd*
689	*P. comestica x P. persica*	root	+	white	*P. fluorescens*
690	*P. comestica x P. persica*	root	+	white	*P. fluorescens*
691	*P. domestica*	leaf	+	white	*P. fluorescens*
692	*P. domestica*	leaf	-	orange	*E. herbicola*
693	*P. domestica*	leaf	-	orange	*E. herbicola*
694	*P. domestica*	leaf	-	orange	*E. herbicola*
695	*P. domestica*	root	+	white	*P. fluorescens*
696	*P. domestica*	root	+	white	*P. fluorescens*
697	*P. domestica*	root	-	orange	*E. herbicola*
698	*P. domestica*	root	+	white	*P. fluorescens*
699	*P. persica x P. dulcis*	leaf	+	white	*P. fluorescens*
700	*P. persica x P. dulcis*	leaf	+	white	*P. fluorescens*
701	*P. persica x P. dulcis*	leaf	-	orange	*E. herbicola*
702	*P. persica x P. dulcis*	leaf	+	white	*P. fluorescens*
703	*P. persica x P. dulcis*	root	+	white	*P. fluorescens*
704	*P. persica x P. dulcis*	root	-	orange	*E. herbicola*
705	*P. persica x P. dulcis*	root	-	orange	*E. herbicola*
706	*P. persica x P. dulcis*	root	-	white	*nd*
707	*P. persica x p. davidiana*	leaf	+	white	*P. fluorescens*
708	*P. persica x p. davidiana*	leaf	-	orange	*E. herbicola*
709	*P. persica x p. davidiana*	leaf	-	orange	*E. herbicola*
710	*P. persica x p. davidiana*	leaf	+	white	*P. fluorescens*
711	*P. persica x p. davidiana*	leaf	+	white	*P. fluorescens*
712	*P. persica x p. davidiana*	leaf	-	orange	*E. herbicola*
713	*P. persica x p. davidiana*	root	+	white	*P. fluorescens*
714	*P. persica x p. davidiana*	root	+	white	*P. fluorescens*
715	*P. persica x p. davidiana*	root	-	white	*nd*
716	*P. persica x P. belsiana*	leaf	-	orange	*E. herbicola*
717	*P. persica x p. davidiana*	root	-	orange	*E. herbicola*

(Continue)

Isolate	Plant species	Organ	Fl[x]	Colony[y]	Species referred[z]
718	*P. dulcis x P. persica*	root	-	orange	*E. herbicola*
719	*P. persica x P. amygdalus*	leaf	-	white	*nd*
720	*P. persica x P. amygdalus*	leaf	+	white	*P. fluorescens*
721	*P. persica x P. amygdalus*	leaf	-	orange	*E. herbicola*
722	*P. persica x P. amygdalus*	root	-	orange	*E. herbicola*
723	*P. persica x P. amygdalus*	root		white	*nd*
724	*P. persica x P. amygdalus*	root	-	white	*nd*
725	*P. persica x P. amygdalus*	root	+	white	*P. fluorescens*
726	*P. persica x P. amygdalus*	root	-	orange	*E. herbicola*
727	*P. persica x P. dulcis*	leaf	-	white	*nd*
728	*P. persica x P. dulcis*	leaf	+	white	*P. fluorescens*
729	*P. persica x P. dulcis*	leaf	+	white	*P. fluorescens*
730	*P. persica x P. dulcis*	leaf	-	orange	*E. herbicola*
731	*P. persica x P. dulcis*	root	+	white	*P. fluorescens*
732	*P. persica x P. dulcis*	root	+	white	*P. fluorescens*
733	*P. persica x P. dulcis*	root	+	white	*P. fluorescens*
734	*P. persica x P. dulcis*	root	-	orange	*E. herbicola*
735	*P. dulcis x P. persica*	leaf	+	white	*P. fluorescens*
736	*P. dulcis x P. persica*	root	-	white	*nd*
737	*P. dulcis x P. persica*	leaf	-	orange	*E. herbicola*
738	*P. dulcis x P. persica*	leaf	+	white	*P. fluorescens*
739	*P. dulcis x P. persica*	root	+	white	*P. fluorescens*
740	*P. dulcis x P. persica*	root	-	orange	*E. herbicola*
741	*P. dulcis x P. persica*	root	+	white	*P. fluorescens*
742	*P. dulcis x P. persica*	root	+	white	*P. fluorescens*
743	*P. dulcis x P. persica*	root	+	white	*P. fluorescens*
744	*P. dulcis x P. persica*	leaf	+	white	*P. fluorescens*
745	*P. dulcis x P. persica*	leaf	-	orange	*E. herbicola*
746	*P. dulcis x P. persica*	leaf	+	white	*P. fluorescens*
747	*P. dulcis x P. persica*	leaf	+	white	*P. fluorescens*
748	*P. dulcis x P. persica*	root	-	white	*nd*
749	*P. dulcis x P. persica*	root	+	white	*P. fluorescens*
750	*P. dulcis x P. persica*	root	-	orange	*E. herbicola*
751	*P. dulcis x P. persica*	root	+	white	*P. fluorescens*
752	*P. domestica*	leaf	-	white	*nd*
753	*P. domestica*	leaf	+	white	*P. fluorescens*
754	*P. domestica*	leaf	+	white	*P. fluorescens*
755	*P. domestica*	leaf	+	white	*P. fluorescens*
756	*P. domestica*	root	-	orange	*E. herbicola*
757	*P. domestica*	root	-	white	*nd*
758	*P. domestica*	root	+	white	*P. fluorescens*
759	*P. insititia*	leaf	+	white	*P. fluorescens*
760	*P. dulcis x P. persica*	root		white	*nd*
761	*P. insititia*	leaf	+	white	*P. fluorescens*
762	*P. insititia*	leaf	+	white	*P. fluorescens*
763	*P. insititia*	root	-	white	*nd*
764	*P. insititia*	root	-	orange	*E. herbicola*
765	*P. insititia*	root	-	white	*nd*
766	*P. insititia*	root	-	blanca	*nd*
767	*P. insititia*	root	+	blanca	*P. fluorescens*
768	*P. domestica*	leaf	-	taronja	*E. herbicola*
769	*P. domestica*	leaf	-	blanca	*nd*
770	*P. domestica*	leaf	+	blanca	*P. fluorescens*
771	*P. domestica*	leaf	+	blanca	*P. fluorescens*
772	*P. domestica*	root	-	taronja	*E. herbicola*
773	*P. domestica*	root	-	blanca	*nd*
774	*P. domestica*	root	+	blanca	*P. fluorescens*
775	*P. domestica*	root	+	blanca	*P. fluorescens*
776	*P. domestica*	root	-	taronja	*E. herbicola*
777	*P. domestica*	root	+	blanca	*P. fluorescens*
778	*P. domestica*	root	-	taronja	*E. herbicola*
779	*P. domestica*	root	-	blanca	*nd*
780	*P. domestica*	root	-	taronja	*E. herbicola*
781	*P. persica x P. dulcis*	root	+	blanca	*P. fluorescens*
782	*P. domestica*	root	+	blanca	*P. fluorescens*
783	*P. domestica*	substratum	-	blanca	*nd*
784	*P. domestica*	substratum	-	blanca	*nd*
785	*P. persica x P. dulcis*	substratum	-	taronja	*E. herbicola*
786	*P. domestica*	substratum	+	blanca	*P. fluorescens*
787	*P. domestica*	substratum	-	blanca	*nd*
788	*P. domestica*	substratum	+	blanca	*P. fluorescens*
789	*P. domestica*	substratum	+	blanca	*P. fluorescens*
790	*P. persica x P. amygdalus*	leaf	-	taronja	*E. herbicola*
791	*P. persica x P. amygdalus*	leaf	+	blanca	*P. fluorescens*
792	*P. persica x P. amygdalus*	leaf	+	blanca	*P. fluorescens*

(Continue)

Isolate	Plant species	Organ	Fl[x]	Colony[y]	Species referred[z]
793	*P. persica x P. amygdalus*	leaf	+	blanca	*P. fluorescens*
794	*P. persica x P. amygdalus*	root	+	blanca	*P. fluorescens*
795	*P. persica x P. amygdalus*	root	-	taronja	*E. herbicola*
796	*P. persica x P. amygdalus*	root	+	blanca	*P. fluorescens*
798	*P. domestica*	leaf	+	blanca	*P. fluorescens*
799	*P. domestica*	leaf	+	blanca	*P. fluorescens*
800	*P. domestica*	leaf	+	blanca	*P. fluorescens*
801	*P. domestica*	leaf	-	blanca	*nd*
802	*P. persica x P. belsiana*	leaf	-	taronja	*E. herbicola*
803	*P. persica x P. belsiana*	leaf	-	taronja	*E. herbicola*
804	*P. persica x P. belsiana*	leaf	+	blanca	*P. fluorescens*
805	*P. persica x P. dulcis*	root	-	taronja	*E. herbicola*
806	*P. persica x P. dulcis*	root	+	blanca	*P. fluorescens*
807	*P. persica x P. dulcis*	root	+	blanca	*P. fluorescens*
808	*P. persica x P. dulcis*	root	+	blanca	*P. fluorescens*
809	*P. persica x P. dulcis*	root	+	blanca	*P. fluorescens*
810	*P. domestica*	leaf	+	blanca	*P. fluorescens*
811	*P. domestica*	root	+	blanca	*P. fluorescens*
812	*P. domestica*	root	+	blanca	*P. fluorescens*
813	*P. persica x P. dulcis*	root	+	blanca	*P. fluorescens*
814	*P. persica x P. davidiana*	root	+	blanca	*P. fluorescens*
815	*P. domestica*	root	+	blanca	*P. fluorescens*
816	*P. persica x P. Belsiana*	leaf	+	blanca	*P. fluorescens*
817	*P. avium*	root	+	blanca	*P. fluorescens*
818	*P. persica x P. Belsiana*	leaf	+	blanca	*P. fluorescens*

[x]Fl, fluorescence production on KB agar under ultraviolet light (366 nm). + = fluorescent colonies; - = no fluorescent colonies.
[y]colony: colony colour on LB agar.
[z]nd: specie non-determined.

ANNEX 6

List of isolates obtained by the enrichment procedure according to organism, source, organ, host species, cultivar, efficacy in the biocontrol of the infections caused by *E. amylovora* and *P. expansum*, antagonism against *E. amylovora* in GA and KB, antagonism against *P. syringae* in GA and KB, antagonism against *Stemphylium vesicarium* in GA and PDA, and antagonism against *P. expansum* in GA.

Isolate	Organism	Source	Organ	Host species	Cultivar	bcea	bcpe	AeaGA	AeaKB	ApsGA	ApsKB	AsvGA	AsvPDA	ApeGA
2e	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Passe Crassane	87.5	10.9	-	+	-	-	-	+	-
3e	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Passe Crassane	75	39.5	-	-	+	-	-	+	-
6e	yeast	Cassa de la Selva	leaf	*Pyrus communis*	Passe Crassane	-	5.8	+	-	+	-	+	-	+
7e	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Passe Crassane	50	31.9	-	-	-	-	-	-	-
8e	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Passe Crassane	0	0	-	-	-	-	-	+	-
10e	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Passe Crassane	100	44.7	-	-	+	-	-	-	-
11e	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Passe Crassane	37.5	35.4	-	-	-	-	-	+	-
13e	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Passe Crassane	100	26.3	-	+	-	-	-	-	-
14e	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Passe Crassane	62.5	27.4	-	-	+	-	-	+	-
16e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	General Leclerc	62.5	28.4	-	-	+	-	-	+	-
17e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	General Leclerc	-	11.4	-	-	-	-	-	+	-
20e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	General Leclerc	28.1	7.6	-	-	-	-	-	-	-
21e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	General Leclerc	93.8	83.9	-	-	+	-	-	+	-
22e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	General Leclerc	31.3		-	-	-	-	-	+	-
23e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	General Leclerc	37.5	4.8	-	+	+	+	+	-	+
25e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	General Leclerc	81.3	26.7	-	-	+	-	-	+	-
26e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	General Leclerc	43.8	10	-	+	+	+	+	-	+
27e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	General Leclerc	56.3	0	-	+	+	-	+	-	+
28e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	50	32.9	-	-	-	-	-	+	-
29e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	87.5	8.7	-	+	+	-	-	+	-
30e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	62.5	5.6	-	-	-	-	-	+	-
31e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	37.5	0	-	-	-	-	-	+	-
32e	yeast	Cassa de la Selva	fruit	*Pyrus communis*	Conference	59.4	83.4	-	-	-	-	-	-	-
33e	yeast	Cassa de la Selva	fruit	*Pyrus communis*	Conference	50	56.9	-	-	-	-	-	-	-
36e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	53.1	1.7	-	-	-	-	-	+	+
38e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	78.1	14	+	+	-	-	+	-	-

(Continue)

Code	Organism	Source	Organ	Host species	Cultivar	bcea	bcpe	AeaGA	AeaKB	ApsGA	ApsKB	AsvGA	AsvPDA	ApeGA
38e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	78.1	14	+	+	-	-	+	-	+
39e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	12.5	1.7	+	+	-	-	+	-	+
40e	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	62.5	16.2	-	-	-	-	+	-	+
41e	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	50	46.8	-	-	-	-	-	+	-
42e	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	37.5	34.2	-	-	-	-	-	+	-
44e	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	-	15.8	-	-	-	-	-	+	-
45e	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	62.5	40.4	-	-	-	-	-	+	-
47e	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	87.5	5.8	-	+	-	+	-	+	+
49e	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	87.5	16.2	-	-	-	-	+	+	-
50e	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	37.5	54.4	-	-	-	-	-	-	-
51e	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	78.1	25.7	-	-	-	-	+	+	-
52e	fungus	Riudellots	fruit	*Pyrus communis*	Passe Crassane	42.9	64.8	-	-	-	-	-	-	-
53e	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	100	4.5	+	+	-	-	+	+	+
54e	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	75	11.9	+	+	-	-	+	+	+
55e	fungus	Riudellots	fruit	*Pyrus communis*	Passe Crassane	71.4	77.8							
56e	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	84.4	14.3	+	-	-	-	+	+	+
58e	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	62.5	7.1	-	-	+	-	-	+	-
59e	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	75	9.9	+	-	+	-	-	+	-
60e	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	75	22	-	-	-	-	-	+	-
61e	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	18.8	13.2	-	-	+	-	-	+	+
62e	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	100	41.2	-	+	-	-	-	-	-
63e	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	87.5	0	+	-	+	-	-	+	+
64e	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	87.5	6.8	+	-	+	-	-	+	-
65e	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	100	11.4	-	+	-	+	-	+	-
66e	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	0	9.4	-	+	-	+	+	-	-
67e	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	40.6	22.1	-	-	-	-	-	-	+
68e	fungus	Riudellots	fruit	*Pyrus communis*	Conference	75	65.4	-	-	-	-	-	-	-
69e	yeast	Riudellots	fruit	*Pyrus communis*	Conference	20.8	83.6	-	-	-	-	-	+	-
70e	fungus	Riudellots	fruit	*Pyrus communis*	Conference	28.6	65	-	-	-	-	-	-	+
71e	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	84.4	19.7	-	-	-	-	-	+	+
72e	yeast	Riudellots	fruit	*Pyrus communis*	Conference	12.5	82.9	-	-	-	-	-	+	-
73e	yeast	Riudellots	fruit	*Pyrus communis*	Conference	62.5	46.5	-	-	-	-	-	+	+

(Continue)

Code	Organism	Source	Organ	Host species	Cultivar	bcea	bcpe	AeaGA	AeaKB	ApsGA	ApsKB	AsvGA	AsvPDA	ApeGA
74	fungus	Riudellots	fruit	*Pyrus communis*	Conference	40.6	59.7	-	-	-	-	-	-	+
75	yeast	Riudellots	fruit	*Pyrus communis*	Conference	50	51.2	-	-	-	-	-	-	-
77	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	37.5	50.9	-	+	+	+	-	+	-
78	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	0	52	-	+	+	+	-	+	-
79	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	50	64.9	-	+	+	+	-	+	-
82	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	100	46.4	-	+	-	+	-	+	-
83	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	62.5	7.4	-	-	-	-	-	+	+
84	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	87.5	7.9	-	+	-	+	-	-	-
85	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	87.5	10	+	+	+	+	-	+	-
86	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	53.1	1	+	+	+	+	-	+	+
87	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	93.8	1.7	+	+	+	+	-	+	+
88	yeast	Cassa de la Selva	leaf	*Pyrus communis*	Comice	75	71.2	-	+	-	-	-	+	-
89	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	100	18.1	-	+	-	-	-	+	+
90	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	0	80.7	+	+	-	-	-	+	-
91	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	87.5	30.8	-	+	+	+	-	-	-
92	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	25	57.3	-	-	-	+	-	-	-
93	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	87.5	6.2	-	+	+	+	-	-	-
94	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	87.5	6	-	+	-	+	-	-	-
95	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	100	7.1	-	+	+	+	-	-	-
96	fungus	Cassa de la Selva	leaf	*Pyrus communis*	Comice	75	70.5	-	-	-	-	-	-	+
97	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	75	17.5	-	+	+	+	-	-	-
98	fungus	Cassa de la Selva	leaf	*Pyrus communis*	Comice	37.5	69.5	-	-	-	-	-	-	+
102	bacteri	Cassa de la Selva	leaf	*Pyrus communis*	Comice	100	51.2	+	+	+	+	-	+	-
104	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	50	32.7	+	-	+	-	-	+	+
105	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	71.9	44.4	-	-	-	-	-	+	-
106	yeast	Riudellots	leaf	*Pyrus malus*	Cardinal	50	45.6	-	-	-	-	-	-	-
107	yeast	Riudellots	leaf	*Pyrus malus*	Cardinal	25	46.9	-	-	-	-	-	-	-
109	fungus	Riudellots	leaf	*Pyrus malus*	Cardinal	-	0	-	-	-	-	-	+	-
110	yeast	Riudellots	leaf	*Pyrus malus*	Cardinal	37.5	39.7	-	-	-	-	-	-	+
111	fungus	Riudellots	leaf	*Pyrus malus*	Cardinal	50	51.9	-	-	-	-	-	-	+
113	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	87.5	74.2	+	+	+	-	+	+	-
112	yeast	Cassa de la Selva	fruit	*Pyrus communis*	Comice	87.5	47.8							

(Continue)

Code	Organism	Source	Organ	Host species	Cultivar	bcea	bcpe	AeaGA	AeaKB	ApsGA	ApsKB	AsvGA	AsvPDA	ApeGA
116	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	50	52.3	+	-	+	-	-	+	+
117	yeast	Cassa de la Selva	fruit	*Pyrus communis*	Comice	37.5	33.5	-	+	-	+	-	-	-
118	yeast	Cassa de la Selva	fruit	*Pyrus communis*	Comice	62.5	36.3	-	+	-	+	-	-	-
119	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	84.4	9.7	+	-	+	-	-	+	-
120	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	50	100	-	-	-	-	-	-	+
122	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	87.5	90.7	-	-	+	-	-	-	+
123	fungus	Cassa de la Selva	fruit	*Pyrus communis*	Comice	87.5	51.5	-	-	-	+	-	+	-
125	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	75	100	-	-	-	-	-	+	-
126	fungus	Cassa de la Selva	fruit	*Pyrus communis*	Conference	62.5	54.4	-	-	-	+	-	+	-
127	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	50	7.4							
128	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	37.5	61.3	-	-	-	+	-	+	-
130	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	100	19.8	+	+	+	+	-		+
131	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	75	100	+	+	+	+	+	+	-
132	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	100	92.6	+	+	+	+	+	+	-
133	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	87.5	19.1	-	+	-	+	-		-
134	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	75	22.3	+	+	-	+	-		-
135	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	0	9.5	-	-	-	-	-	-	-
136	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	75	17.7	+	+	-	+	+	+	+
137	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Comice	62.5	0	+	+	-	+	+	+	+
138	bacteri	Cassa de la Selva	fruit	*Pyrus malus*	Cardinal	50	14.74	-	-	-	+	-	-	-
140	bacteri	Cassa de la Selva	fruit	*Pyrus malus*	Cardinal	87.5	27.2	-	-	-	-	-	-	-
142	yeast	Cassa de la Selva	fruit	*Pyrus malus*	Cardinal	37.5	62.1	-	-	-	-	-	-	+
143	yeast	Cassa de la Selva	fruit	*Pyrus malus*	Cardinal	87.5	72.9							
144	fungus	Cassa de la Selva	fruit	*Pyrus malus*	Cardinal	25	84							
145	fungus	Cassa de la Selva	fruit	*Pyrus malus*	Cardinal	65.6	56.6	-	+	-	+	-	+	-
148	bacteri	Cassa de la Selva	fruit	*Pyrus malus*	Cardinal	37.5	23.9	+	+	+	-	-	+	-
150	bacteri	Cassa de la Selva	leaf	*Cydonia oblonga*	--------	68.8	50.6	-	-	-	+	-	-	-
151	bacteri	Cassa de la Selva	leaf	*Cydonia oblonga*	--------	21.8	28	-	-	-	+	-	+	-
156	bacteri	Cassa de la Selva	leaf	*Cydonia oblonga*	--------	96.9	62.7	+	+	-	+	-	+	+
157	fungus	Cassa de la Selva	leaf	*Pyrus communis*	Comice	62.5	53.2	+	+	+	+	-	-	-
158	fungus	Cassa de la Selva	leaf	*Cydonia oblonga*	--------	87.5	8.3	-	-	-	+	-	+	-
160	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	9.4	69.6	+	-	-	+	-	-	-

(Continue)

Code	Organism	Source	Organ	Host species	Cultivar	bcea	bcpe	AeaGA	AeaKB	ApsGA	ApsKB	AsvGA	AsvPDA	ApeGA
162	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	6.3	4.2	-	-	-	-	-	+	+
165	yeast	Cassa de la Selva	fruit	*Pyrus communis*	Conference	37.5	40.7	-	-	-	-	-	+	-
167	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	0	16.8	+	+	+	+	-	-	-
169	yeast	Cassa de la Selva	fruit	*Pyrus communis*	Conference	68.8	55.9	-	-	-	-	-	+	-
170	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	28.1	59.8	-	-	-	+	-	+	-
172	yeast	Cassa de la Selva	fruit	*Pyrus communis*	Conference	37.5	35.3	-	-	-	-	-	+	-
173	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	75	38.8							
174	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	75	15.6							
175	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	37.5	4.1	-	+	+	+	+	+	-
176	yeast	Riudellots	fruit	*Pyrus communis*	Passe Crassane	50	56.7							
178	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	46.9	42.4	-	+	-	-	+	+	-
179	bacteri	Riudellots	fruit	*Pyrus communis*	Passe Crassane	37.5	18.3	-	+	-	+	+	+	-
181	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	75	24.8	+	-	+	-	-	+	-
182	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	25	11							
183	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	53.1	27.1	+	+	+	-	-	+	+
184	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	50	29.1							
185	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	75	17.6	+	-	+	-	-	+	+
186	yeast	Riudellots	leaf	*Pyrus malus*	Cardinal	37.5	37.4							
187	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	62.5	24.5	+	-	-	-	-	+	-
189	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	30	44.3	-	+	-	-	-	-	-
195	bacteri	Riudellots	fruit	*Pyrus malus*	Cardinal	12.5	20.4	-	+	-	-	-	-	+
196	bacteri	Riudellots	fruit	*Pyrus malus*	Cardinal	28.1	43.5	-	+	-	-	-	-	-
197	bacteri	Riudellots	leaf	*Cydonia oblonga*	--------	12.5	5.52	-	+	-	+	-	-	+
198	bacteri	Riudellots	leaf	*Cydonia oblonga*	--------	12.5	18.9	+	-	+	+	-	+	-
199	bacteri	Riudellots	leaf	*Cydonia oblonga*	--------	-	34.7	-	+	-	+	-	+	-
200	bacteri	Riudellots	leaf	*Cydonia oblonga*	--------	9.4	0	-	-	-	-	-	-	-
201	bacteri	Riudellots	leaf	*Cydonia oblonga*	--------	6.3	2.9	+	+	+	+	-	+	-
202	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	100	25.9	-	-	-	-	-	+	-
203	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	75	30.2	-	-	-	-	-	+	-
205	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	31.25	20.1	-	+	-	+	-	+	-
206	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	25	23.7	-	+	-	+	-	+	-
207	yeast	Cassa de la Selva	fruit	*Pyrus communis*	Conference	37.5	76.9							

(Continue)

Code	Organism	Source	Organ	Host species	Cultivar	bcea	bcpe	AeaGA	AeaKB	ApsGA	ApsKB	AsvGA	AsvPDA	ApeGA
208	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	75	34.4	-	-	-	-	-	+	-
209	yeast	Cassa de la Selva	fruit	*Pyrus communis*	Conference	37.5	43.4							
210	bacteri	Cassa de la Selva	fruit	*Pyrus communis*	Conference	100	31.7	-	-	-	-	-	+	-
211	yeast	Cassa de la Selva	fruit	*Pyrus communis*	Conference	43.8	23	-	-	-	-	-	+	-
213	yeast	Girona Fruits	fruit	*Pyrus communis*	Conference	25	63	-	-	-	-	-	+	-
214	yeast	Girona Fruits	fruit	*Pyrus malus*	Golden Smothee	37.5	56.5	-	-	-	-	-	-	-
215	yeast	Girona Fruits	fruit	*Pyrus malus*	Golden Smothee	75	52.1	-	-	-	-	-	-	-
216	yeast	Girona Fruits	fruit	*Pyrus malus*	Golden Smothee	25	60.6							
217	yeast	Girona Fruits	fruit	*Pyrus malus*	ERO	62.5	58.6	-	-	-	-	-	+	-
218	bacteri	Girona Fruits	fruit	*Pyrus malus*	ERO	25	27.2							
219	yeast	Girona Fruits	fruit	*Pyrus malus*	ERO		67.9	-	-	-	-	-	-	-
220	yeast	Girona Fruits	fruit	*Pyrus malus*	ERO	65.6	51.2	-	-	+	-	-	-	-
222	bacteri	Girona Fruits	fruit	*Pyrus communis*	Conference	0	28.3	-	+	-	+	-	-	-
224	bacteri	Girona Fruits	fruit	*Pyrus communis*	Conference	0	37.5	-	-	-	+	-	+	-
225	bacteri	Girona Fruits	fruit	*Pyrus communis*	Conference	12.5	29.6	-	-	-	-	-	-	-
226	bacteri	Girona Fruits	fruit	*Pyrus communis*	Conference	12.5	17.1	-	+	-	+	-	+	-
227	bacteri	Girona Fruits	fruit	*Pyrus communis*	Conference	12.5	19.7	-	+	+	+	-	-	-
228	bacteri	Girona Fruits	fruit	*Pyrus communis*	Conference	34.4	0	-	+	-	+	-	-	-
229	yeast	Girona Fruits	fruit	*Pyrus malus*	Golden Smothee	140.6	55.1	-	-	-	-	-	-	-
230	bacteri	Girona Fruits	fruit	*Pyrus malus*	Golden Smothee	87.5	24.1	-	-	-	-	-	-	+
231	bacteri	Girona Fruits	fruit	*Pyrus malus*	Golden Smothee	31.25	4.9	-	-	-	-	-	-	-
232	bacteri	Girona Fruits	fruit	*Pyrus malus*	ERO	34.4	33.3	-	+	+	+	-	-	-
233	bacteri	Girona Fruits	fruit	*Pyrus malus*	ERO	12.5	23.6	-	-	-	-	-	-	-
234	bacteri	Girona Fruits	fruit	*Pyrus malus*	ERO	28.1	6.7	-	-	-	-	-	+	+
235	bacteri	Girona Fruits	fruit	*Pyrus malus*	ERO	18.75	34.1	-	+	-	+	-	+	+
236	bacteri	Girona Fruits	fruit	*Pyrus malus*	ERO	43.75	21.4	-	+	+	+	-	-	-
237	bacteri	Girona Fruits	fruit	*Pyrus communis*	Ercolini	100	16	+	-	+	+	-	-	-
238	yeast	Girona Fruits	fruit	*Pyrus communis*	Ercolini	50	47.3	-	-	-	-	-	+	-
239	bacteri	Girona Fruits	fruit	*Pyrus communis*	Ercolini	12.5	14.7	-	-	-	-	-	+	-
240	yeast	Girona Fruits	fruit	*Pyrus communis*	Conference	62.5	37.3	-	-	-	-	-	+	-
242	yeast	Girona Fruits	fruit	*Pyrus malus*	Golden Smothee	28.1	67	-	-	-	-	-	+	-
245	yeast	Girona Fruits	fruit	*Pyrus malus*	ERO	25	63.2	-	-	-	-	-	+	-

(Continue)

Code	Organism	Source	Organ	Host species	Cultivar	bcea	bcpe	AeaGA	AeaKB	ApsGA	ApsKB	AsvGA	AsvPDA	ApeGA
246	yeast	Girona Fruits	fruit	*Pyrus communis*	Ercolini	56.2	73.4	-	-	-	-	-	+	-
247	bacteri	Riudellots	fruit	*Pyrus communis*	Conference	18.75	14.5	-	-	-	+	-	+	-
248	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	50	48	-	-	-	-	-	+	-
249	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	40.62	10.1	-	+	-	-	-	-	-
250	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	40.62	13.3	+	+	+	+	+	-	+
251	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	25	15.4	-	+	-	+	-	+	-
252	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	28.1	23.6	+	+	-	+	-	-	-
253	yeast	Girona Fruits	fruit	*Pyrus communis*	Ercolini	18.8	60.4	+	+	-	-	-	-	+
254	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	87.5	27.2	+	+	-	-	-	-	-
255	yeast	Girona Fruits	fruit	*Pyrus communis*	Ercolini	25	80.7	-	-	-	-	-	+	-
256	bacteri	Girona Fruits	fruit	*Pyrus communis*	Ercolini	56.3	18.5	-	-	-	-	-	+	-
258	bacteri	Riudellots	leaf	*Pyrus malus*	Cardinal	31.3	15.3	+	+	-	-	-	-	-
259	bacteri	Pals	leaf	*Vitis vinifera*	--------	100	54.4							
260	bacteri	Pals	leaf	*Vitis vinifera*	--------	46.9	53.8	-	+	-	-	-	+	-
261	bacteri	Pals	leaf	*Vitis vinifera*	--------	0	12	-	+	-	+	-	+	+
262	bacteri	Pals	leaf	*Vitis vinifera*	--------	12.5	13.7	-	+	-	+	-	+	+
263	bacteri	Pals	leaf	*Vitis vinifera*	--------	0	29.1	-	+	-	+	-	+	+
264	bacteri	Pals	leaf	*Vitis vinifera*	--------	34.4	15.9	-	+	-	+	-	+	+
265	bacteri	Pals	leaf	*Vitis vinifera*	--------	18.8	28.7	-	+	-	+	-	+	+
266	bacteri	Pals	leaf	*Vitis vinifera*	--------	28.1	13.1	-	+	-	+	-	+	+
267	yeast	Pals	leaf	*Vitis vinifera*	--------	37.5	67.4	+	+	-	-	-	+	+
268	bacteri	Pals	leaf	*Vitis vinifera*	--------	28.1	26.7	+	+	-	+	-	+	-
269	yeast	Pals	leaf	*Vitis vinifera*	--------	62.5	84.5							
270	bacteri	Pals	leaf	*Vitis vinifera*	--------	-	17.1							
271	yeast	Pals	leaf	*Vitis vinifera*	--------	50	65.6	+	-	-	+	-	+	+
272	yeast	Pals	leaf	*Vitis vinifera*	--------	18.8	37.5	+	+	-	+	-	+	+
273	bacteri	Pals	leaf	*Vitis vinifera*	--------	87.5	89.4	-	+	-	+	-	+	+
274	bacteri	Pals	leaf	*Vitis vinifera*	--------	25	24.2	-	+	-	+	-	+	+
275	bacteri	Pals	leaf	*Pyrus malus*	--------	0	27.6	+	+	-	-	-	-	-
276	bacteri	Pals	leaf	*Pyrus malus*	--------	59.4	61.6	+	-	-	-	-	-	-
279	bacteri	Pals	leaf	*Medicago sativa*	--------	21.9	40	-	+	-	+	-	+	+
280	bacteri	Pals	leaf	*Medicago sativa*	--------	25	32.7							

(Continue)

Code	Organism	Source	Organ	Host species	Cultivar	bcea	bcpe	AeaGA	AeaKB	ApsGA	ApsKB	AsvGA	AsvPDA	ApeGA
281	bacteri	Pals	leaf	*Medicago sativa*	--------	9.4	36.1	-	-	-	+	-	+	+
282	bacteri	Pals	leaf	*Prunus avium*	--------	37.5	36.7	-	+	-	+	-	+	+
283	bacteri	Pals	leaf	*Prunus avium*	--------	100	25	-	-	-	-	-	+	-
284	bacteri	Pals	leaf	*Prunus avium*	--------	87.5	12.1	-	-	-	-	-	+	-
285	bacteri	Pals	leaf	*Prunus avium*	--------	25	25	-	-	-	-	-	-	-
286	bacteri	Pals	leaf	*Prunus avium*	--------	37.5	46.4	-	+	-	-	-	+	-
287	bacteri	Pals	leaf	*Prunus avium*	--------	37.5	30.4	-	+	-	+	-	+	-
288	bacteri	Pals	leaf	*Prunus avium*	--------	87.5	23.4	-	-	-	-	-	-	-
290	bacteri	Pals	leaf	*Prunus avium*	--------	12.5	21.8	-	+	-	+	-	+	+
291	bacteri	Pals	leaf	*Prunus avium*	--------	18.8	36.6	-	+	-	+	-	+	-
292	bacteri	Pals	leaf	*Prunus avium*	--------	21.9	24.8	+	+	-	-	-	+	-
293	bacteri	Pals	leaf	*Prunus avium*	--------	34.4	21.6	+	+	-	+	-	+	-
294	bacteri	Pals	leaf	*Prunus avium*	--------	25	47.1	-	+	-	+	-	+	-
295	yeast	Pals	leaf	*Prunus avium*	--------	50	38.6							
298	yeast	Pals	leaf	*Prunus avium*	--------	37.5	5.7	-	-	-	-	-	+	-
299	bacteri	Pals	leaf	*Prunus avium*	--------	34.4	21.4	-	+	-	+	-	+	-
300	bacteri	Pals	leaf	*Medicago sativa*	--------	0	8.6	+	+	-	+	-	+	-
301	bacteri	Pals	leaf	*Medicago sativa*	--------	75	33.3	-	+	-	-	-	+	-
303	bacteri	Pals	leaf	*Medicago sativa*	--------	31.3	33.9	-	+	-	+	-	+	-
304	yeast	Pals	leaf	*Medicago sativa*	--------	62.5	30.6	-	+	-	+	-	+	+
305	bacteri	Pals	leaf	*Medicago sativa*	--------	75	17	-	+	-	+	-	+	+
306	bacteri	Pals	leaf	*Medicago sativa*	--------	9.4	25.8	-	+	-	+	-	+	+
307	yeast	Pals	leaf	*Medicago sativa*	--------	62.5	60.6	-	+	-	+	-	+	+
308	bacteri	Pals	leaf	*Medicago sativa*	--------	15.6	22.1	-	+	-	+	-	+	-
309	bacteri	Pals	leaf	*Medicago sativa*	--------	9.4	11.9	+	+	-	+	-	+	-
311	bacteri	Pals	leaf	*Medicago sativa*	--------	100	29.9	+	-	-	+	-	+	+
313	bacteri	Pals	leaf	*Medicago sativa*	--------	100	26.8							
315	bacteri	Pals	leaf	*Vitis vinifera*	--------	37.5	74.1	+	+	-	+	-	+	+
316	bacteri	Pals	leaf	*Vitis vinifera*	--------	87.5	38.3	+	+	-	+	-	+	+
317	bacteri	Pals	leaf	*Vitis vinifera*	--------	100	24.1	+	+	-	+	-	+	+
318	bacteri	Pals	leaf	*Vitis vinifera*	--------	100	33.1	-	+	-	+	-	+	+
319	yeast	Pals	leaf	*Vitis vinifera*	--------	68.8	0	-	+	-	+	-	+	-

(Continue)

Code	Organism	Source	Organ	Host species	Cultivar	bcea	bcpe	AeaGA	AeaKB	ApsGA	ApsKB	AsvGA	AsvPDA	ApeGA
321	bacteri	Pals	leaf	*Vitis vinifera*	--------	100	21.1	-	+	-	+	-	+	+
323	bacteri	Pals	leaf	*Vitis vinifera*	--------	75	25.9	+	+	-	+	-	+	+
324	bacteri	Pals	leaf	*Vitis vinifera*	--------	87.5	23.2	-	-	-	-	-	+	-
325	bacteri	Pals	leaf	*Vitis vinifera*	--------	0	45.6	+	+	-	-	-	+	+
326	bacteri	Pals	leaf	*Vitis vinifera*	--------	100	27.2	+	+	-	+	-	+	+
327	bacteri	Pals	leaf	*Vitis vinifera*	--------	100	43.4	+	+	-	+	-	+	+
329	bacteri	Pals	leaf	*Vitis vinifera*	--------	87.5	18	+	+	-	+	-	+	+
330	bacteri	Pals	leaf	*Vitis vinifera*	--------	96.9	42.8	-	+	-	+	-	+	+
331	bacteri	Pals	leaf	*Vitis vinifera*	--------	78.1	25.9	-	+	-	+	-	+	+
333	fungus	Pals	leaf	*Vitis vinifera*	--------	56.3	73.9	-	+	-	+	-	+	+
337	bacteri	Pals	leaf	*Vitis vinifera*	--------	87.5	27	-	+	-	+	-	+	+
340	bacteri	Pals	leaf	*Pyrus malus*	--------	81.25	32.9	-	+	-	+	-	+	+
341	bacteri	Pals	leaf	*Pyrus malus*	--------	50	48.7	-	-	-	+	-	+	-
342	bacteri	Pals	leaf	*Pyrus malus*	--------	100	41.6	+	-	-	+	-	+	+
343	bacteri	Pals	leaf	*Pyrus malus*	--------	62.5	45.5	+	+	-	+	-	+	+
344	yeast	Pals	leaf	*Pyrus malus*	--------	62.5	-	-	+	-	+	-	+	+
345	bacteri	Pals	leaf	*Pyrus malus*	--------	100	21.2	-	+	-	+	-	+	+
346	bacteri	Pals	leaf	*Prunus avium*	--------	87.5	31.1	-	+	-	+	-	+	+
347	bacteri	Pals	leaf	*Prunus avium*	--------	87.5	44.1	+	+	-	+	-	+	+
348	bacteri	Pals	leaf	*Prunus avium*	--------	100	23	-	+	-	+	-	+	+
349	bacteri	Pals	leaf	*Vitis vinifera*	--------	100	0	-	+	-	+	-	+	+
350	bacteri	Pals	leaf	*Vitis vinifera*	--------	62.5	30.6	+	+	-	+	-	+	+
351	bacteri	Pals	leaf	*Vitis vinifera*	--------	75	33.6	+	-	-	+	-	+	+
352	bacteri	Pals	leaf	*Vitis vinifera*	--------	100	17.5	+	+	-	+	-	+	+
355	yeast	Pals	leaf	*Vitis vinifera*	--------	37.5	65.1	-	-	-	+	-	+	+
357	bacteri	Pals	leaf	*Vitis vinifera*	--------	75	40	+	-	-	+	-	+	+
358	bacteri	Pals	leaf	*Prunus avium*	--------	100	42	+	+	-	+	-	+	-

bcea, biocontrol of *E. amylovora* in immature pear fruits; bcpe , biocontrol of *P. expansum* in apple fruits; AeaGA, AeaKB, antagonism against *E. amylovora* in GA and KB, respectively; ApsGA, ApsKB, antagonism against *P. syringae* in GA and KB, respectively; AsvGA, AsvPDA, antagonism against *S. vesicarium* in GA and PDA, respectively; ApePDA, antagonism against *P. expansum* in PDA.

ANNEX 7

List of strain selected by activity criteria according to species, source, organ, host species, bbcontrol against *E. amylovora* in immature pear fruits, cyanide, indolacetic acid, siderophores, chitinases, 2,4-diacetylphlorogucinol, phenazine-1-carboxylic acid production.

Code	Species	Source	Organ	Host species	bcea	HCN	IAA	Sid	Chit	Phl	PCA
207	*P.fluorescens*	Girona	root	*Scorpiurus subvillosus*	77.8	-	-	+	-	-	-
214	*P.fluorescens*	Girona	root	*Anthylis montana*	77.8	+	-	+	-	-	-
227	*P.fluorescens*	Girona	root	*Pyrus communis*	75	-	-	+	-	-	-
229	*P.fluorescens*	Girona	root	*P.communis*	80.6	-	-	+	-	-	-
230	*P.fluorescens*	Girona	root	*P.communis*	88.9	-	+	+	-	-	-
232	*P.fluorescens*	Girona	root	*P.communis*	88.9	+	-	+	-	-	-
234	*P.fluorescens*	Girona	root	*P.communis*	88.9	+	-	+	-	-	-
254	*P.fluorescens*	Mas Badia	root	*P.malus* (M-25)	91.7	+	-	+	-	-	-
272	*P.fluorescens*	Coll de Merolla	root	*Carex*	80.6	+	-	+	-	-	-
297	*P.fluorescens*	Setcases	root	*Graminia*	75	+	-	+	-	-	-
307	*P.fluorescens*	Setcases	root	*Sarotamnus*	77.8	-	+	+	-	-	-
308	*P.fluorescens*	Coll de Merolla	root	*Trigonella*	86.1	+	-	+	-	-	-
317	*P.fluorescens*	Girona	root	*Nicoliana tabacum*	16.7	-	-	+	-	+	-
323	*P.fluorescens*	Fornells	root	*Helianthus annuus*	80.6	+	-	+	-	-	-
327	*P.fluorescens*	Fornells	root	*Lactuca sativa*	68	+	-	+	-	-	-
328	*P.fluorescens*	Fornells	root	*Lactuca sativa*	86.1	+	-	+	+	-	-
337	*P.fluorescens*	Fornells	root	*Zea mays*	80.6	-	-	+	-	-	-
338	*P.fluorescens*	Calonge	leaf	*P.communis* (cv. general Leclerc)	100	-	-	+	-	-	-
340	*P.fluorescens*	Lleida	bud	*Prunus domestica*	77.8	-	-	+	+	-	-
346	*P.fluorescens*	-	-	-	77.8	-	-	+	-	-	-
351	*P.fluorescens*	Girona	-	*P.communis*	90.6	-	-	+	-	-	-
354	*P.fluorescens*	Alava	bud	*Vitis vitifera*	72.2	-	-	+	-	-	-
363	*P.fluorescens*	-	-	-	77.8	-	-	+	-	-	-
371	*P.fluorescens*	Girona	bud	*P.communis*	33.3	-	-	+	-	-	-
372	*P.fluorescens*	Mas Badia	leaf	*P.communis*	75	-	-	+	-	-	-
374	*P.fluorescens*	Girona	bud	*P.communis*	86.1	-	-	+	-	-	-
375	*P.fluorescens*	Girona	bud	*P.communis*	81.2	-	-	+	-	-	-
377	*P.fluorescens*	Girona	bud	*P.communis*	71.9	-	-	+	-	-	-
381	*E.herbicola*	Banyoles	flower	*Veronia persica*	75	-	+	+	-	-	-

(Continue)

Code	Species	Source	Organ	Host species	bcea	HCN	IAA	Sid	Chit	Phl	PCA
434	*E.herbicola*	Casadella	bud	*P.communis* (cv. Conference)	91.7	-	+	+	-	-	-
437	*E.herbicola*	Sant Iscle	bud	*Cydonia oblonga*	75	-	-	-	-	-	-
438	*E.herbicola*				100	-	+	+	-	-	-
439	*E.herbicola*	Cassa de la Selva	flower	*Prunus persica*	100	-	+	+	-	-	-
440	*E.herbicola*	Gualta	bud	*Prunus persica*	83.3	-	-	+	-	-	-
442	*E.herbicola*	St. Pere Pescador	bud	*Pyrus malus* (cv. Top Red)	86.1	-	+	+	-	-	-
443	*E.herbicola*	LArmentera	bud	*Prunus avium*	88.9	-	+	+	-	-	-
452	*E.herbicola*	Viladomat	flower	*Prunus persica*	77.8	-	+	+	-	-	-
454	*E.herbicola*	Vilamacolum	flower	*Prunus persica*	83.3	-	+	+	-	-	-
455	*E.herbicola*	Armentera	flower	*Prunus persica*	94.4	-	+	+	-	-	-
457	*E.herbicola*	St. Pere Pescador	bud	*Pyrus malus* (cv. Starking)	75	-	+	+	-	-	-
460	*E.herbicola*	Viladomat	bud	*Prunus avium*	80.6	-	-	+	-	-	-
461	*E.herbicola*	Armentera	flower	*Prunus domestica*	77.8	-	+	+	-	-	-
465	*P.fluorescens*	St. Iscle	bud	*Cydonia oblonga*	75	-	-	+	-	-	-
471	*E.herbicola*	Casadella	flower	*Pyrus communis*	77.8	-	-	+	-	-	-
475	*E.herbicola*	Sibina	bud	*Prunus persica*	77.8	-	+	+	-	-	-
476	*E.herbicola*	Sibina	bud	*Prunus persica*	83.3	-	-	+	-	-	-
480	*E.herbicola*	Vilamacolum	flower	*Prunus persica* (cv.Baby Golden)	86.1	-	+	+	-	-	-
481	*E.herbicola*	Mas Oller	bud	*Pyrus malus* (cv. Royal Gala)	52.8	-	-	+	-	-	-
482	*E.hervicola*	Roman	bud	*Pyrus communis* (cv.Conference)	72.2	-	+	+	-	-	-
490	*E.herbicola*	Armentera	bud	*Prunus avium*	80.6	-	-	+	-	-	-
497	*E.herbicola*	Tarragona	bud	*Prunus avium*	72.2	-	+	+	-	-	-
516	*E.herbicola*	Sibina	bud	*Pyrus communis*(cv.P. Crassane)	100	-	+	+	-	-	-
519	*E.herbicola*	Vidal	bud	*Pyrus communis*(cv.P. Crassane)	77.8	-	+	+	-	-	-
520	*E.herbicola*	Cassa de la Selva	bud	*Pyrus communis*	77.8	-	+	+	-	-	-
525	*E.herbicola*	Tarragona (El Catllar)	flower	*Prunus persica*	91.7	-	+	+	-	-	-
527	*E.herbicola*	Mas Oller	bud	*Pyrus communis*(cv.P. Crassane)	88.9	-	+	+	-	-	-
528	*E.herbicola*	Mas Oller	bud	*Pyrus communis*(cv.P. Crassane)	83.3	-	+	+	-	-	-
538	*P.fluorescens*	Can Calonge	bud	*Pyrus communis* (cv. G. Leclerc)	75	-	-	+	-	-	-
541	*E.herbicola*	Calonge	bud	*Prunus avium*	88.9	-	+	+	-	-	-
542	*E.herbicola*	Calonge	flower	*Pyrus communis*	91.7	-	+	+	-	-	-
547	*E.herbicola*	St. Antoni de Calonge	flower	*Prunus domestica*	77.8	-	+	+	-	-	-
549	*E.herbicola*	St. Antoni de Calonge	leaf	*Prunus persica*	86.1	-	+	+	-	-	-
551	*E.herbicola*		leaf	*Prunus amygdalus*	88.9	-	+	+	-	-	-

(Continue)

Code	Species	Source	Organ	Host species	bcea	HCN	IAA	Sid	Chit	Phl	PCA
552	*E.herbicola*	St. Joan de Palamos	leaf	*Prunus amygdalus*	91.7	-	+	+	-	-	-
554	*E.herbicola*	Calonge	flower	*Prunus persica*	75	-	-	+	-	-	-
560	*E.herbicola*	Mas Oller	leaf	*Crataegus*	94.4	-	+	+	-	-	-
566	*E.herbicola*	Mas Oller	leaf	*Crataegus*	77.8	-	-	+	-	-	-
567	*E.herbicola*	Can Calonge	leaf	*Pyrus communis* (cv. G.Leclerc)	88.9	-	+	+	-	-	-
583	*P.fluorescens*	Llagostera	leaf	*Crataegus*	88.9	-	-	+	-	-	-
587	*P.fluorescens*	Llagostera	flower	*Crataegus*	72.2	-	-	+	-	-	-
594	*E.herbicola*	Mas Badia	flower	*Pyrus malus*	77.8	-	+	+	-	-	-
597	*P.fluorescens*	Llagostera	leaf	*Crataegus*	100	-	-	+	-	-	-
599	*P.fluorescens*	Llagostera	flower	*Crataegus*	91.7	-	-	+	-	-	-
602	*P.fluorescens*	Llagostera	leaf	*Crataegus*	88.2	-	-	+	-	-	-

bcea, biocontrol against *E. amylovora* in immature pear fruits; HCN, cyanide; IAA, indolacetic acid; SID, siderophores; CHI, chitinases; phl, 2,4-diacetylphloroglucinol; PCA, phenazine-1-carboxylic acid.

ANNEX 8

List of isolates in function of the efficacy in the inhibition of infections by *E. amylovora* CUCM273 in immature pear fruits. The fruits were treated by immersion in the isolate suspensions 24 h before of pathogen inoculation at 10^7 CFU mL^{-1}

Efficacy intervals[x]	**Strain**													
VA	10e	13e	21e	53e	62e	65e	82e	87e	89e	95e	102e	130e	132e	156e
	202e	210e	237e	259e	283e	311e	313e	317e	318e	321e	326e	327e	330e	342e
	343e	345e	348e	349e	352e	357e	358e	372	405	412	438	455	460	481
	538	549	594	660	664	684	697	702	708	720	734	738	CHA0	St2128
A	2e	3e	25e	29e	38e	47e	49e	51e	54e	55e	56e	59e	60e	63e
	64e	68e	71e	84e	85e	88e	91e	93e	94e	96e	97e	105e	112e	113e
	119e	122e	123e	125e	131e	133e	134e	136e	140e	143e	158e	173e	174e	181e
	185e	203e	208e	215e	230e	254e	273e	284e	288e	301e	305e	316e	323e	324e
	329e	331e	337e	340e	346e	347e	351e	357e	308	338	374	375	377	381
	404	434	437	454	457	465	480	490	516	520	525	528	551	552
	554	566	567	583	587	597	599	658	659	661	668	671	675	677
	681	689	692	695	698	703	710	717	719	722	724	733	736	737
	739	741	749	750	754	756	776	784	787	789	795	797	802	803
	806	811	817	818	BI915	Ps278	St2131							
MA	7e	14e	16e	26e	27e	28e	30e	32e	33e	36e	37e	40e	41e	45e
	52e	58e	67e	73e	74e	75e	79e	83e	86e	104e	106e	111e	116e	118e
	120e	126e	127e	137e	138e	145e	150e	157e	169e	176e	178e	183e	184e	187e
	189e	211e	214e	217e	218e	220e	229e	236e	238e	240e	246e	248e	250e	256e
	260e	269e	271e	276e	295e	304e	307e	319e	333e	341e	343e	344e	350e	272
	307	317	323	328	351	354	363	395	397	403	439	440	442	443
	452	461	471	475	482	527	541	542	547	560	602	651	652	653
	654	657	662	663	667	670	674	678	679	680	683	686	687	688
	690	691	693	694	699	700	701	704	707	711	718	721	723	725
	728	730	731	740	742	743	745	746	748	751	753	755	758	760
	761	762	766	768	771	772	777	781	786	788	794	800	807	810
	815	jbr170	St378	st844										
SA	11e	20e	22e	23e	31e	42e	50e	69e	70e	77e	92e	98e	107e	110e
	117e	128e	142e	144e	148e	151e	165e	170e	172e	175e	179e	182e	186e	196e
	205e	206e	207e	209e	213e	216e	228e	231e	323e	234e	242e	245e	249e	251e
	252e	255e	258e	264e	266e	267e	268e	274e	279e	280e	282e	285e	286e	287e
	292e	293e	294e	298e	299e	303e	315e	355e	207	214	227	229	230	254
	297	337	371	476	497	519	655	666	672	673	682	696	712	712
	714	716	726	729	732	738	747	757	759	767	770	775	782	783
	791	792	798	799	804	808	809	812	813	814	816	St2126		
NA	8e	39e	61e	66e	72e	78e	90e	135e	153e	160e	162e	167e	195e	197e
	198e	200e	201e	222e	224e	225e	226e	227e	233e	235e	239e	247e	253e	261e
	262e	263e	265e	272e	275e	281e	290e	291e	300e	306e	308e	309e	325e	232
	234	327	340	346	650	665	669	685	706	715	727	744	752	763
	764	765	769	773	774	779	780	790	793	796	801	805	Ps15	Jmp1284
	Ps31	Q287	Q487	St2129										

[x]isolates were grouped by the efficacy in the inhibition of infections in immature pear fruits, as very active (100-90 %), active (90-70 %), moderately active (70-40 %), soft active (40-20 %) and not active (20-0 %).

ANNEX 9

List of selected isolates based on colony colour, host specie, plant organ source, efficacy in the inhibition of infection caused by *E. amylovora* CUCM273 in immature pear fruits and selection procedure.

Isolate	Colony colour[x]	Host specie	Plant organ	Efficay[y] (%)	Selection procedure[z]
10e	taronja	*Pyrus communis*	leaf	100.0	Extracts
13e	taronja	*P. communis*	leaf	100.0	Extracts
21e	taronja	*P. communis*	fruit	93.7	Extracts
53e	blanca	*P. communis*	fruit	100.0	Extracts
62e	blanca	*P. communis*	fruit	100.0	Extracts
65e	blanca	*P. communis*	fruit	100.0	Extracts
82e	blanca	*P. communis*	leaf	100.0	Extracts
87e	blanca	*P. communis*	leaf	93.7	Extracts
89e	blanca	unknown	leaf	100.0	Extracts
90e	taronja	unknown	leaf	100.0	Extracts
95e	blanca	unknown	leaf	100.0	Extracts
102e	blanca	unknown	leaf	100.0	Extracts
130e	taronja	*P. communis*	fruit	100.0	Extracts
132e	taronja	*P. communis*	fruit	100.0	Extracts
156e	taronja	*Cydonia oblonga*	leaf	96.8	Extracts
173e	blanca	*P. communis*	fruit	75.0	Extracts
202e	taronja	*P. communis*	fruit	100.0	Extracts
210e	taronja	*P. communis*	fruit	100.0	Extracts
230e	taronja	*Pyrus malus*	fruit	87.5	Extracts
237e	taronja	*P. communis*	fruit	100.0	Extracts
259e	vermella	*Vitis vinifea*	leaf	100.0	Extracts
283e	taronja	*Prunus avium*	leaf	100.0	Extracts
301e	vermella	*Medicago sativa*	leaf	75.0	Extracts
311e	vermella	*M. sativa*	leaf	100.0	Extracts
313e	vermella	*M. sativa*	leaf	100.0	Extracts
317e	vermella	*Vitis vinifera*	leaf	100.0	Extracts
318e	vermella	*V. vinifera*	leaf	100.0	Extracts
321e	vermella	*V. vinifera*	leaf	100.0	Extracts
326e	vermella	*V. vinifera*	leaf	100.0	Extracts
327e	vermella	*V. vinifera*	leaf	100.0	Extracts
330e	vermella	*V. vinifera*	leaf	96.8	Extracts
342e	vermella	*P. malus*	leaf	100.0	Extracts
343e	vermella	*P. malus*	leaf	96.8	Extracts
345e	vermella	*P. malus*	leaf	100.0	Extracts
348e	vermella	*P. avium*	leaf	100.0	Extracts
349e	vermella	*V. vinifera*	leaf	100.0	Extracts
352e	vermella	*V. vinifera*	leaf	100.0	Extracts
357e	vermella	*V. vinifera*	leaf	100.0	Extracts
358e	vermella	*P. avium*	leaf	100.0	Extracts
338	taronja	*P. communis*	leaf	87.5	Antagonists
372	blanca	*P. communis*	leaf	93.7	Antagonists
405	taronja	*P. communis*	bud	100.0	Antagonists
412	taronja	*P. malus*	bud	90.6	Antagonists
438	taronja	unknown	unknown	100.0	Antagonists
455	taronja	*Prunus persica*	flower	96.8	Antagonists
460	taronja	*P. avium*	bud	100.0	Antagonists
481	taronja	*P. malus*	bud	100.0	Antagonists
538	blanca	*P. communis*	bud	100.0	Antagonists
549	taronja	*P. persica*	leaf	100.0	Antagonists
594	taronja	unknown	unknown	100.0	Antagonists
660	taronja	*P. persica x Prunus davidiana*	root	100.0	Specie
664	blanca	*P. avium*	leaf	100.0	Specie
684	blanca	*Prunus domestica x P. persica*	leaf	90.6	Specie
697	taronja	*P. domestica*	root	100.0	Specie
702	blanca	*P. persica x Prunus dulcis*	leaf	93.7	Specie
708	taronja	*P.persica x P. davidiana*	leaf	100.0	Specie
720	blanca	*P. persica x P. davidiana*	leaf	100.0	Specie
734	blanca	*P. persica x P. dulcis*	root	96.8	Specie
735	blanca	*P. dulcis x P. persica*	leaf	93.7	Specie
784	blanca	unknown	substrate	87.5	Specie
818	blanca	*P.persica x P.Belsiana x P. domestica*	leaf	87.5	Specie

[x]Colony colour in LB.

[y]Efficacy in the inhibition of infections by *E. amylovora* CUCM273 at 10^7 CFU ml^{-1} in immature pears fruits.

[z]Isolation procedure: extracts, isolates from vegetal extracts with capacity to inhibit *E. amylovora* infections in immature pear fruits; antagonists, isolates with capacity to inhibit infections by *E. amylovora* in immature pear fruits; specie, isolates selected to belong to *P. fluorescens* or *E. herbicola*.