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IMPROVEMENT OF STRATEGIES FOR THE MANAGEMENT OF FIRE BLIGHT (ERWINIA AMYLOVORA). EVALUATION AND OPTIMIZATION OF PHYSICAL AND CHEMICAL CONTROL METHODS, AND USE OF DECISION SUPPORT SYSTEMS

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TESI DOCTORAL

IMPROVEMENT OF STRATEGIES FOR THE MANAGEMENT OF FIRE BLIGHT (*Erwinia amylovora*). EVALUATION AND OPTIMIZATION OF PHYSICAL AND CHEMICAL CONTROL METHODS, AND USE OF DECISION SUPPORT SYSTEMS

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

Lídia Ruz i Estévez

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CERTIFIQUEN:

Que la llicenciada en Biologia Lídia Ruz Estévez ha dut a terme, sota la seva direcció, el treball amb el títol "Improvement of strategies for the management of fire blight (*Erwinia amylovora*). Evaluation and optimization of physical and chemical control methods, and use of decision support systems", 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 9 de setembre de 2003.

Vist-i-plau directors de la Tesi

Dra. Concepció Moragrega Garcia

Dr. Isidre Llorente Cabratosa

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SUMMARY

Fire blight, caused by the bacterium *Erwinia amylovora,* is a serious disease of rosaceous plants that affects fruit trees such as pear, apple or quince, and ornamental plants with great commercial and economic interest. The disease is spread and well distributed in all temperate regions of the world. In Spain, where the disease is non endemic, fire blight was first detected in 1995 in the North of the country (Euskadi) and later, several new outbreaks have appeared in other locations that have been properly eradicated. Control of fire blight is very slightly effective in affected plants and is based on measures to avoid the spread of pathogen, and the introduction of disease in non-endemic regions.

The low effectiveness of the currently available methods for fire blight control, and the present situation of fire blight in Spain require an improvement in strategies to prevent and control the disease. In order to prevent the entry of the fire blight pathogen in disease-free regions strategies based on thermotherapy such as quarantine or prophylactic methods may be useful. Moreover, as chemical control is currently the most effective control method in countries or regions where the disease is well established, the improvement and optimization of chemical control methods, and the evaluation of new compounds like benzothiadiazole and phosphonate derivatives, are unavoidable. Additionally, the knowledge of the effect of these compounds on *E. amylovora*-host plant interaction is of particular interest to elucidate the mode of action of these products. Finally, in order to guide prospections and to support chemical control measures, available fire blight risk prediction models must be evaluated under Spanish weather conditions.

In this work, thermotherapy has been evaluated as a method for eradication of *E. amylovora* from symptomless propagating plant material. The effect of heat type, temperature and exposure time on plant viability and on survival of *E. amylovora* was determined. It has been demonstrated that heat is a viable method for eradicating *E. amylovora* from the propagation material of the pear. Almost all rosaceous species and cultivars maintained under moist conditions survived 7 hours at 45 ºC and up to 3 hours at 50 ºC, while more than 1 hour of exposure at 50 ºC under dry heat injured plants and reduced shooting. Moreover, the time required to reduce the *E. amylovora* populations to non-detectable level (50 cfu mL^{-1}) was up to 80 min at 45 ºC and up to 60 min at 50 ºC. However, 60 min at 45 ºC or 30 min at 50 ºC were enough to reduce epiphytic *E. amylovora* population on pear budwoods to non-detectable level (5 x 10^2 cfu g^{-1} f.w.). Endophytic populations of *E. amylovora* in pear plants were eliminated after 8 hours at 45 ºC or more than 3 hours at 50 ºC.

The improvement of chemical control of fire blight has been carried out by comparing and optimizing the effectiveness of copper derivatives, antibiotics and plant defense inducers (phosphonates and benzothiadiazole). Different formulations, doses and strategies of application of

copper derivatives and a copper-mancozeb mixture were tested. Copper formulations and copper-mancozeb mixtures similarly controlled the disease in pear plants, and for all products the maximal reduction of disease was obtained at the highest copper-dose (3 g Cu L^{-1}). The protective application of copper derivatives was more effective than the preventive and curative strategies in control of disease. Phosphonate derivatives and benzothiadiazole were effective in fire blight control in pear and apple, under laboratory, greenhouse and field conditions. Their efficacy depended on the dose and the time interval between product application and bacteria inoculation, since several days were required to completely induce plant defenses. Plant defense inducers reduced disease levels to 40-60%. The minimal time intervals to achieve the best control of disease were 5 days for fosetyl-Al, and 7 days for ethephon and benzothiadiazole, and the optimal doses of fosetyl-Al and benzothiadiazole were 3.72 g HPO_3^{2-} L^{-1} and 150 mg a.i. L^{-1}, respectively. The efficacy of fosetyl-Al and benzothiadiazole in fire blight control was improved when consecutively sprayed (combined strategy) with a half-reduced dose of antibiotics. Although the mixed strategy is more practical and easier to apply in the orchard than the combined one, the best level of fire blight control was achieved with the combined strategy. In addition, phosphonates and benzothiadiazole had very low *in vitro* antibacterial activity against 14 strains of plant-pathogenic and non-plant-pathogenic bacteria. This low *in vitro* antibacterial activity together with their efficacy in disease control in pear suggests their future use in fire blight control as an alternative to conventional compounds.

The effect of benzothiadiazole and phosphonates in *Erwinia amylovora*-pear interaction was analyzed at histological, ultrastructural and molecular level. In particular, structural changes in pear leaf tissues produced by *E. amylovora* infection, and ultrastructural modifications after product application were studied and compared with the effects of copper and streptomycin. In addition, molecular studies of pear defense genes induced by benzothiadiazole were included in an attempt to elucidate the mechanism of action of benzothiadiazole. Our results indicated that the early site of tissue colonization by *E. amylovora* after pear leaf inoculation was the vascular system rather than the intercellular space and the bacteria can colonize the intercellular spaces of the parenchyma after disrupting the xylem vessels. Neither benzothiadiazole, nor fosetyl-Al, nor ethephon induced structural changes in pear leaf tissues 7 days after their application. However, after *E. amylovora* inoculation structural cell disorganization was observed in fosetyl-Al and ethephon-sprayed plants, while in benzothiadiazole-sprayed plants these tissue alterations were delayed. Among some apple molecular markers of SAR only chitinase and β-1,3-glucanase were induced by benzothiadiazole and ethephon respectively in pear.

Preliminary studies of fire blight risk prediction models in the Northeast of Spain were performed by Montesinos and Llorente (1999) and Llorente *et al.* (2002). Following these studies in the present work fire blight risk maps based on historical weather data from Northeastern Spain have been made. Two predictive models (Maryblyt and Cougarblight) were evaluated in an orchard naturally

affected by fire blight in Spain, to determine the accuracy of the predictions. The combined BRS-Powell model and the modified BIS95 model were also evaluated. Results showed two clearly differentiated geographical areas with high and low fire blight risk. The high risk region was located around the Ebro river valley (between Huesca and Zaragoza) and Lleida, whereas the low risk area included Girona and Barcelona. Differences were found between the two models used, the frequency of high risk predicted by the modified BIS95 being higher than expected. Maryblyt and Cougarblight models were evaluated in an orchard in 2002, where the previous season a major fire blight outbreak took place. Results indicated two infection risk periods with the Maryblyt model and four with Cougarblight. However no outbreak had occurred in the orchard. Maryblyt and Cougarblight are easy models to use, but their implementation in disease management programs must be evaluated and validated for more seasons and in areas where the disease is present.

RESUMEN

El fuego bacteriano es una enfermedad que afecta a plantas de la familia de la rosáceas, causada por la bacteria *Erwinia amylovora*. Su rango de huéspedes incluye árboles frutales, como el peral, el manzano o el membrillero, y plantas ornamentales de gran interés comercial y económico. Actualmente, la enfermedad se ha dispersado y se encuentra ampliamente distribuida en todas las zonas de clima templado del mundo. En España, donde la enfermedad no es endémica, el fuego bacteriano se detectó por primera vez en el 1995 en el norte del país (Euskadi) y posteriormente, han aparecido varios focos en otras localizaciones, que han sido convenientemente erradicados. El control del fuego bacteriano, es muy poco efectivo en plantas afectadas por la enfermedad, de manera que se basa en medidas encaminadas a evitar la dispersión del patógeno y la introducción de la enfermedad en regiones no endémicas.

Debido a la baja efectividad de los métodos actualmente disponibles para el control del fuego bacteriano y a la situación de la enfermedad en España es necesario realizar estudios que permitan conocer y determinar las mejores estrategias para prevenir y controlar la enfermedad. Para prevenir la entrada d'*E. amylovora* en zonas libres de la enfermedad, es necesario desarrollar estrategias basadas en la termoterapia como método de cuarentena. Además, como que el control químico, es actualmente el método más eficaz de control en países o regiones donde la enfermedad está bien introducida, su mejora y optimización, así como la evaluación de nuevos compuestos como el benzotiadiazol y los derivados de los fosfonatos en el control del fuego bacteriano es inevitable. Adicionalmente, el análisis del efecto de estos compuestos en la interacción *E. amylovora*-huésped podría ser interesante. Finalmente, un sistema de soporte de decisiones para guiar los trabajos de prospección y las medidas de control químico, y la elaboración de mapas de riesgo de fuego bacteriano es necesario.

En el presente trabajo, la termoterapia se ha avaluado como método de erradicación d'*E. amylovora* de material vegetal de propagación asintomático. Se ha determinado el efecto del tipo de calor, la temperatura y el tiempo de exposición, en la viabilidad del material vegetal y en la supervivencia de *E. amylovora in vitro* y en el material vegetal. Se ha demostrado que la termoterapia es un método viable para erradicar *E. amylovora* de material de propagación. Casi todas las especies y variedades de rosáceas mantenidas en condiciones de humedad sobreviven 7 horas a 45 ºC y más de 3 horas a 50 ºC, mientras que más de 1 hora de exposición a 50 ºC con calor seco producía daños en el material vegetal y reducía la brotación. Por otro lado, el tiempo necesario para reducir las poblaciones de *E. amylovora* a niveles inferiores al nivel de detección (50 ufc mL^{-1}) fueron superiores a 80 min a 45 ºC y superiores a 60 min a 50 ºC. Tratamientos de 60 min a 45 ºC o 30 min a 50 ºC fueron suficientes para reducir la población epífita de *E. amylovora* a niveles no detectables (5 x 10^2 ufc g^{-1} p.f.) en ramas de peral. Mientras que las

poblaciones endófitas de *E. amylovora* en plantas de peral, pueden ser eliminadas después de 8 horas a 45 ºC o más de 3 horas a 50 ºC con calor húmedo.

La mejora del control químico del fuego bacteriano se ha realizado comparando y optimizando la eficacia de los derivados de cobre, antibióticos y los inductores de defensas de las plantas (fosfonatos y benzotiadiazol). Se han avaluado diferentes formulaciones, dosis y estrategias de aplicación de los derivados de cobre. Para mejorar la eficacia del cobre en el control del fuego bacteriano se han avaluado mezclas de cobre y mancozeb. Las formulaciones de cobre y las mezclas de cobre-mancozeb controlaron de manera similar la enfermedad en peral, y para todos los productos, la máxima reducción de la enfermedad se obtuvo a la dosis de cobre más elevada (3 g Cu L^{-1}). La aplicación protectiva de los derivados de cobre resultó más efectiva en el control de la enfermedad que la preventiva y la curativa. Los derivados de los fosfonats y el benzotiadiazol fueron efectivos en el control del fuego bacteriano en peral y manzano, tanto en condiciones de laboratorio, como de invernadero y campo. Su eficacia depende de la dosis y del intervalo de tiempo entre la aplicación del producto y la inoculación de la bacteria, ya que son necesarios varios días para inducir completamente las defensas de la planta. Los inductores de defensas de las plantas reducen los niveles de la enfermedad hasta el 40-60%. Los intervalos de tiempo mínimos para conseguir el mejor control de la enfermedad fueron 5 días para el fosetil-Al, y 7 días para el etefon y el benzotiadiazol, y las dosis óptimas para el fosetil-Al y el benzotiadiazol fueron 3.72 g HPO_3^{2-} L^{-1} y 150 mg i.a. L^{-1}, respectivamente. Se mejora la eficacia del fosetil-Al y del benzotiadiazol en el control del fuego bacteriano, cuando se combinan con los antibióticos a la mitad de la dosis de éstos últimos. Aunque la estrategia de mezclar productos es más práctica y fácil de llevar a cabo en campo, que la estrategia de combinar productos, el mejor nivel de control de la enfermedad se consigue con la estrategia de combinar productos. Además, los fosfonatos y el benzotiadiazol presentaron un reducida actividad antibacteriana *in vitro* frente a las 14 cepas de bacterias fitopatógenas y no fitopatógenas evaluadas. La baja actividad antibacteriana *in vitro,* junto con la moderada eficacia en el control de la enfermedad permitiría su futuro uso en el control del fuego bacteriano como alternativa a los productos convencionales.

Se analizó a nivel histológico, ultrastructural y molecular el efecto del benzotiadiazol y de los fosfonatos en la interacción *Erwinia amylovora*-peral. En particular, se estudiaron los cambios estructurales producidos en los tejidos de la hoja de peral por la infección de *E. amylovora*, y las modificaciones ultrastructurales después de la aplicación de los productos comparándolos con los efectos del cobre y la estreptomicina. Por otro lado, se incluyeron estudios moleculares sobre la inducción de genes de defensa de peral por el benzotiadiazol, para intentar determinar el mecanismo de acción de este producto. Nuestros resultados en hojas de peral indican que inicialmente la colonización por *E. amylovora* después de la inoculación se produce por el sistema vascular más que por el espacio intercelular, aunque la bacteria puede colonizar los espacios intercelulares del parénquima después de romper las vesículas del xilema. Ni el benzotiadiazol, ni

el fosetil-Al, ni el etefon indujeron cambios estructurales en los tejidos de peral 7 días después de su aplicación. No obstante, después de la inoculación de *E. amylovora* se observó en las plantas tratadas con fosetil-Al y etefon una desorganización estructural celular, mientras que en las plantas tratadas con benzotiadiazol estas alteraciones tisulares fueron retardadas. Entre algunos de los marcadores moleculares de SAR en manzano, sólo la quitinasa y la β-1,3-glucanasa fueron inducidas en peral por el benzotiadiazol y el etefon, respectivamente.

Montesinos y Llorente (1999) y Llorente *et al.* (2002) habían realizado estudios preliminares sobre modelos de predicción de riesgo del fuego bacteriano en el nordeste de España. Continuando estos estudios, en el presente trabajo se han elaborado los mapas de predicción de riesgo del fuego bacteriano en base a datos meteorológicos históricos del nordeste español. Se han avaluado dos modelos (Maryblyt, Cougarblight) en un campo en España afectado por la enfermedad, para determinar la precisión de las predicciones. Se utilizaron dos modelos para elaborar el mapa de riesgo, el BRS-Powell combinado y el BIS95 modificado. Los resultados mostraron dos zonas con elevado y bajo riesgo de la enfermedad. La zona de elevado riesgo se localizó alrededor de la ribera del río Ebro (entre Huesca y Zaragoza) y Lleida, mientras que la zona de bajo riesgo incluyó las provincias de Girona y Barcelona. Entre los dos modelos utilizados se observaron diferencias, la frecuencia de riesgo elevado con el modelo BIS95 modificado fue superior a la que se podría esperar. La evaluación de los modelos Maryblyt y Cougarblight se realizó en el 2002 en un campo, con un importante foco de la enfermedad la temporada anterior. Los resultados que se obtuvieron indican que el modelo de Maryblyt predeció dos períodos de riesgo de infección y el de Cougarblight cuatro períodos. Sin embargo, no apareció ningún foco de la enfermedad en la finca ese año. Maryblyt y Cougarblight son dos modelos de fácil uso, aunque su implementación en programas de manejo de la enfermedad requiere que sean evaluados y validados durante un período de tiempo más largo y en áreas donde la enfermedad este presente.

RESUM

El foc bacterià és una malaltia que afecta a plantes de la família de la rosàcies, causada pel bacteri *Erwinia amylovora*. El seu rang d'hostes inclou arbres fruiters, com la perera, la pomera o el codonyer, i plantes ornamentals de gran interès comercial i econòmic. Actualment, la malaltia s'ha dispersat i es troba àmpliament distribuïda en totes les zones de clima temperat del món. A Espanya, on la malaltia no és endèmica, el foc bacterià es va detectar per primer cop al 1995 al nord del país (Euskadi) i posteriorment, han aparegut varis focus en altres localitzacions, que han estat convenientment eradicats. El control del foc bacterià, és molt poc efectiu en plantes afectades per la malaltia, de manera que es basa en mesures encaminades a evitar la dispersió del patogen, i la introducció de la malaltia en regions no endèmiques.

Degut a la baixa efectivitat del mètodes actualment disponibles pel control del foc bacterià i per la situació actual de la malaltia a Espanya és necessari realitzar estudis que permetin conèixer i determinar les millors estratègies per prevenir i controlar la malaltia. Per prevenir l'entrada d'*E. amylovora* en zones lliures de la malaltia, és necessari desenvolupar estratègies basades en la termoteràpia com a mètode de quarantena. A més, com que el control químic, és actualment el mètode més efectiu de control en països o regions on la malaltia està ben establerta, la seva millora i optimització, així com l'avaluació de nous compostos com el benzotiadiazol i els derivats dels fosfonats en el control del foc bacterià és inevitable. Addicionalment, analitzar l'efecte d'aquests compostos en la interacció *E. amylovora*-hoste podria ser interessant. Finalment, un sistema de suport de decisions per guiar els treballs de prospecció i les mesures de control químic, i l'elaboració de mapes de risc de foc bacterià són necessàries.

En aquest treball, la termoteràpia ha estat avaluada com a mètode d'eradicació d'*E. amylovora* de material vegetal de propagació asimptomàtic. L'efecte del tipus de calor, la temperatura i el temps d'exposició, en la viabilitat de material vegetal i en la supervivència d'*E. amylovora in vitro* i en el material vegetal han estat determinats. S'ha demostrat que la termoteràpia és un mètode viable d'eradicar *E. amylovora* de material de propagació. Gairebé totes les espècies i varietats de rosàcies mantingudes en condicions d'humitat sobrevivien 7 hores a 45 ºC i més de 3 hores a 50 ºC, mentre que més d'1 hora d'exposició a 50 ºC amb calor seca produïa danys en el material vegetal i reduïa la brotació. Per altra banda, el temps necessari per reduir les poblacions d'*E. amylovora* a nivells inferiors al nivell de detecció (50 ufc mL^{-1}) va ser superior a 80 min a 45 ºC i superior a 60 min a 50 ºC. Tractaments de 60 min a 45 ºC o 30 min a 50 ºC van ser suficients per reduir la població epífita d'*E. amylovora* a nivells no detectables (5 x 10^2 ufc g^{-1} p.f.) en branques de perera. Mentre que les poblacions endòfites d'*E. amylovora* en plantes de perera, poden ser eliminades després de 8 hores a 45 ºC o més de 3 hores a 50 ºC amb calor humida.

La millora del control químic del foc bacterià s'ha realitzat comparant i optimitzant l'eficàcia dels derivats de coure, antibiòtics i els inductors de defensa de les plantes (fosfonats i benzotiadiazol). S'han avaluat diferents formulacions, dosis i estratègies d'aplicació dels derivats de coure. Per millorar l'eficàcia del coure en el control del foc bacterià s'han avaluats barreges de coure i mancozeb. Les formulacions de coure i les barreges de coure-mancozeb van controlar de manera similar la malaltia en perera, i per tots els productes, la màxima reducció de la malaltia es va obtenir a la dosi de coure més elevada (3 g Cu L^{-1}). L'aplicació protectiva dels derivats de coure va resultar més efectiva que la preventiva i la curativa en el control de la malaltia. Els derivats dels fosfonats i el benzotiadiazol són efectius en el control del foc bacterià en perera i pomera, tant en condicions de laboratori, com d'hivernacle i camp. La seva eficàcia depèn de la dosi i de l'interval de temps entre l'aplicació del producte i la inoculació del bacteri, ja que són necessaris varis dies per induir completament les defenses de la planta. Els inductors de defensa de les plantes redueixen els nivells de malaltia fins al 40-60%. Els intervals de temps mínims per aconseguir el millor control de la malaltia van ser 5 dies pel fosetil-Al, i 7 dies per l'etefon i el benzotiadiazol, i les dosis òptimes pel fosetil-Al i el benzotiadiazol van ser 3.72 g HPO_3^{2-} L^{-1} i 150 mg i.a. L^{-1}, respectivament. Es millora l'eficàcia del fosetil-Al i del benzotiadiazol en el control del foc bacterià, quan es combinen amb els antibiòtics a la meitat de la dosi d'aquests últims. Tot i que l'estratègia de barrejar productes és més pràctica i fàcil de dur a terme a camp, que l'estratègia de combinar productes, el millor nivell de control de la malaltia s'aconsegueix amb l'estratègia de combinar productes. A més, els fosfonats i el benzotiadiazol presentaren baixa activitat antibacteriana *in vitro* front les 14 soques de bacteris fitopatògens i no fitopatògens avaluades. Aquesta baixa activitat antibacteriana *in vitro* juntament amb la seva eficàcia en el control de la malaltia podrien fer possible el seu futur ús en el control del foc bacterià com a alternativa als productes convencionals.

Es va analitzar a nivell histològic, ultrastructural i molecular l'efecte del benzotiadiazol i dels fosfonats en la interacció *Erwinia amylovora*-perera. En particular, es van estudiar els canvis estructurals produïts en el teixits de la fulla de perera per la infecció d'*E. amylovora*, i les modificacions ultrastructurals després de l'aplicació dels productes comparant-los amb els efectes del coure i l'estreptomicina. Per altra banda, es van incloure estudis moleculars sobre la inducció de gens de defensa de perera pel benzotiadiazol, per intentar determinar el mecanisme d'acció d'aquest producte. Els nostres resultats en fulles de perera indiquen que inicialment la colonització per *E. amylovora* després de la inoculació va ser pel sistema vascular més que per l'espai intercel·lular, tot i que el bacteri pot colonitzar els espais intercel·lulars del parènquima després de trencar les vesícules del xilema. Ni el benzotiadiazol, ni el fosetil-Al, ni l'etefon van induir canvis estructurals en els teixits de perera 7 dies després de la seva aplicació. No obstant, després de la inoculació d'*E. amylovora* es va observar en plantes tractades amb fosetil-Al i etefon una desorganització estructural cel·lular, mentre que en les plantes tractades amb benzotiadiazol aquestes alteracions tissulars van ser retardades. Entre alguns dels marcadors moleculars de

SAR en pomera, només la quitinasa i la β-1,3-glucanasa van ser induïdes en perera pel benzotiadiazol i l'etefon, respectivament.

Montesinos and Llorente (1999) i Llorente *et al.* (2002) havien realitzat estudis preliminars sobre models de predicció de risc de foc bacterià en el nord-est d'Espanya. Continuant aquests estudis, en el present treball s'han elaborat els mapes de predicció de risc de foc bacterià en base a dades meteorològiques històriques del nord-est espanyol. S'han avaluat dos models (Maryblyt, Cougarblight) en un camp a Espanya afectat per la malaltia, per determinar la precisió de les prediccions. Es van utilitzar dos models per elaborar el mapa de risc, el BRS-Powell combinat i el BIS95 modificat. Els resultats van mostrar dos zones amb elevat i baix risc de la malaltia. La zona d'elevat risc es va localitzar al voltant de la ribera del riu Ebre (entre Osca i Saragossa) i Lleida, mentre que la zona de baix risc va incloure les províncies de Girona i Barcelona. Entre els dos models utilitzats es van observar diferències, la freqüència de risc elevat amb el model BIS95 modificat va ser superior al que caldria esperar. L'avaluació dels models Maryblyt i Cougarblight es va realitzar al 2002 en un camp, on la temporada anterior un important focus de la malaltia havia tingut lloc. Els resultats obtinguts van indicar que el model de Maryblyt va predir dos períodes de risc d'infecció i el de Cougarblight quatre períodes. Tanmateix, cap focus de la malaltia va aparèixer a la finca. Maryblyt i Cougarblight són dos models de fàcil ús, tot i que la seva implementació en programes de maneig de la malaltia requereix que siguin avaluats i validats per un període de temps més llarg i en àrees on la malaltia hi estigui present.

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ABBREVIATIONS

a.i.	Active ingredient
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
BIS95	Billing's integrated system, 1995
BOS	Billing's original system
BRS	Billing's revised system
BTH	Benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester
cfu	Colony-forming units
CHT	Chitinase
CREAF	Centre de recerca ecològica i aplicacions forestals
cv.	Cultivar
Cu	Copper
CYE	Casitone yeast extract
DD	Degree day
DDH	Degree day-hour
DH	Degree hour
DEPC	Diethyl pyrocarbonate
DSS	Decision support system
EC	European community
ED	Effective dose
EF-1α	Elongation factor 1α
ELISA	Enzyme linked immunosorbent assay
f.w.	Fresh weight
GIS	Geographic information system
GLM	General linear models
GLU	β-1,3-glucanase
GMT	Greenwich mean time
h	Hour
IR	Induced resistance
IRS	Infection risk score
KB	King B
L	Liter
LB	Luria Bertani
LT	Lethal thermal
MIC	Minimal inhibitory concentration
min	Minute
NTC	Non-treated control
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase chain reaction
PD	Potential doubling
PR	Pathogenesis-related
pv.	Pathovar
RH	Relative humidity
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SA	Salicylic acid
SAR	Systemic acquired resistance
SAS	Statistical analysis system
U	Units

INTRODUCTION

1. HISTORY AND GEOGRAPHICAL DISTRIBUTION OF FIRE BLIGHT

Fire blight is a bacterial plant disease caused by *Erwinia amylovora* (Burr.) (Winslow *et al.*). This disease affects species of the rosaceous family such as pear, apple, quince and ornamentals (*Pyracantha* spp., *Crataegus* spp., *Cotoneaster* spp., ...). Fire blight has been considered as undoubtedly the oldest, most serious, and perplexing bacterial disease of pomaceous fruit trees (van der Zwet and Keil, 1979). It was first described in 1780. About this disease it has been said that 'there is probably no disease of fruit trees so thoroughly destructive as fire blight' (Waite, 1895 in Thomson, 2000). Recently, it has been published a review by leading researchers from different countries, who have reported that fire blight is the most devastating bacterial disease of apples and pears (Vanneste, 2000). At the moment, the disease is still one of the most serious problems of fruit trees, and it is one of the more studied bacterial diseases. Additionally, fire blight was the first disease shown to be caused by a bacterium (before thought to be caused by insects), and *Erwinia amylovora* was the first plant pathogenic bacterium shown to be spread by insects (Baker, 1971 in Vanneste, 2000).

GEOGRAPHICAL DISTRIBUTION

The disease was first detected in North-America, in the New York State (USA), although nowadays it is spread and well distributed in all temperate regions of the world. In the last century the disease has been reported from 40 countries around the world. In 1919, fire blight was first reported in New Zealand, in 1957 the first focus in Europe (England) was observed, in 1959 the disease entered in South-America, in Chile, and in 1964 in the Nil valley in Egypt (van der Zwet, 1968). The disease has been spread in Europe from North to South and from West to East. In 1966, it was first described in Poland (Sobiczewski and Suski, 1988). Fire blight has also been detected in France and Belgium (1972), Greece (1985), Israel (Zutra, 1986), Turkey (Öktem and Benlioglu, 1988) and Italy (1990). In 1992 the disease was detected in Japan and in some countries in the Middle East (Mazzucchi, 1992). The disease may be present in other countries, but it has not been yet observed or still reported. In Switzerland the fire blight was observed first in 1989 and since 1995 it is steadily spreading (FOAG, 2000). The last countries in Europe where fire blight has been reported for the first time are Austria in 1993 (Keck *et al*., 1996), and Hungary in 1996 (Németh, 1999). However, there are countries considered as free of fire blight, including Spain and Portugal (Figure 1).

In Spain, the first outbreak was detected in 1995, in Euskadi, in cider apple orchards located to few kilometers from the French border (de la Cruz Blanco, 1996; López *et al.,* 1999). In 1996 and 1997, new outbreaks were observed in different regions in the North of the country (Guipuzkoa and Navarra). In 1996, fire blight was detected and eradicated in a nursery located in Segovia (Castilla-

León). Two years later, in 1998, the disease was detected in a nursery in Guadalajara (Castilla-La Mancha), on imported plants from The Netherlands. In the same year it was detected the first outbreak in Lleida (Catalunya). In 1999, a new occurrence of fire blight was observed in Lleida with eight new outbreaks mainly on pear tree orchards, and in a garden in Jaca (Huesca). All these outbreaks were correctly eradicated (DARP, 2000). In 2000 the disease was first detected in La Rioja and in a commercial pear orchard in Zaragoza. In the following year new outbreaks in the same area appeared which were eradicated and in 2002 only few orchards were affected.

Spain is considered under the laws of the European Union as a protected area for fire blight and a monitoring network has been set up under the direction of the Plant Protection Services of each region (Gorris *et al.,* 1996; López *et al.,* 1999).

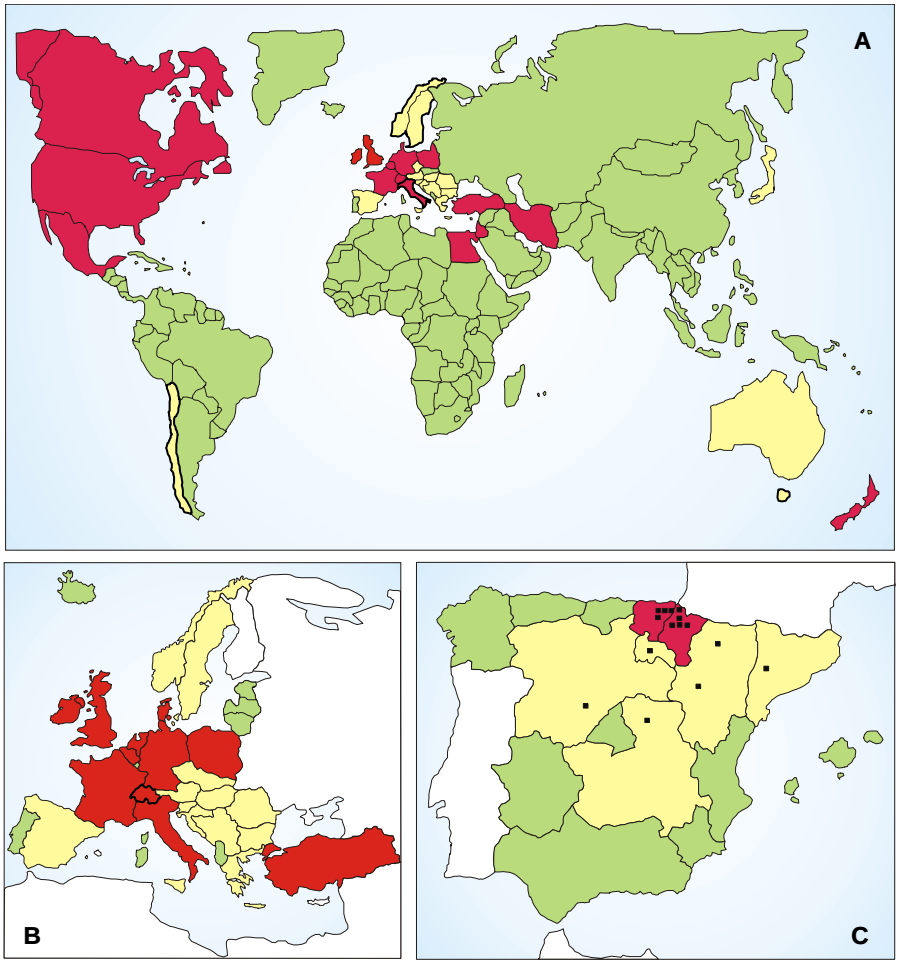

Figure 1. Fire blight distribution in the world (A), in Europe (B) and in Spain (C). ■ Countries or regions where fire blight is widely and early distributed, ■ where is partially distributed, ■ and where has still not been described. ■ Outbreaks appeared in Spain from 1995 to 2002.

From the apparition of the first outbreak in Spain, several management strategies, such as inspection to identify potentially infected plants and an eradication policy, were rapidly implemented to control the spread of the pathogen in the country. The incidence of the disease, if any, is still low in fruit-producing areas. However, considering the rapid progression of the disease in other Mediterranean countries, it is necessary to establish appropriate management strategies to minimize the potential losses.

2. ECONOMIC IMPORTANCE

Fire blight is considered the most damaging disease of pome fruits in some countries of North-America and Europe. It is extremely dangerous for the industry of pear and apple. Fire blight is an economically important disease for several reasons. Firstly, for the range of plant species affected (pear, apple, quince and woody ornamentals) of great commercial importance. Secondly, by the lack of effective control methods. There is still no registered product that can effectively control the fire blight and avoid the disease spread. Furthermore, Vanneste (2000) reported that the economic importance of this disease is likely to increase, because fire blight is still spreading geographically into new apple and pear growing areas, and the new production methods, such as high-density planting, the use of susceptible rootstocks (M.26 and M.9) and new apple cultivars (Gala and Broedurn). Additionally, in some countries (New Zealand) fire blight is not only a problem associated with production (only very occasionally causes losses of flowers in some areas), but also the prohibition of the exportation to countries free of the disease indirectly causes several economic losses (Hale *et al.,* 1996).

The production is strongly reduced when the infection occurs, since the infections normally begin through flowers, affecting finally the fruits. Indeed, a high percentage of plants infected by *E. amylovora* die in a few months. Unlike many other plant diseases, fire blight can cause losses in the current season's crop, and produces permanent damage in an orchard because, once fire blight becomes established throughout one region, it provokes severe infections and serious losses. Accurate estimates of losses due to fire blight are difficult to obtain, but there is no doubt that they range in millions of dollars annually (Table 1).

Among the diseases affecting pome fruit trees such as blossom blast caused by *Pseudomonas syringae* pv. *syringae*, pear and apple scab caused by *Venturia pirina* (anamorph *Fusicladium pyrorum*) and *Venturia inaequalis* (anamorph *Spilocaea pomi)* respectively, and brown spot of pear caused by *Stemphylium vesicarium* (teleomorph *Pleospora allii)*, fire blight is the disease that causes greatest losses in various fruit production regions in Europe (Montesinos and López, 1998).

France and Italy are examples of the recent introduction of the disease. In France, the production of apple and pear is forced to the coexistence with the disease and a cultivar conversion has taken

place with total substitution of the pear sensible cultivar Passe Crassane. In Italy, pear production is concentrated in the region of Emilia-Romagna (Northeast Italy) and the cultivar structure is dominated by very sensible material. This fact has caused a very fast evolution of the disease in the last years, from 30 outbreaks in 1996 to 721 in 1997 (with more than 24,000 ha of pear trees severely infected) and 888 in 1998 (500,000 fruit trees were destroyed due to fire blight) (Calzolari *et al.,* 1999).

Table 1. Losses due to fire blight in susceptible crops in different countries (Bonn and van der Zwet, 2000; Vanneste, 2000; FOAG, 2000)

Year	Country	Losses
2000	USA (Southwest Michigan)	US$42 million
2000	Switzerland	€4.11 million
1998	USA (Northwest)	US$68 million
1998	New Zealand (Hawkes Bay)	NZ$10 million
1997	Italy (Po Valley)	500,000 fruit trees destroyed
1996	Macedonia	US$7 million
1996	Hungary	US$1.1 million
1991	USA (Michigan)	US$3.8 million
1990	Croatia	In 10 years, 1 million of trees destroyed
1988	Greece	300 ha destroyed
1988	Egypt	In 8 years, 50% of trees destroyed

In Spain, the production of pome fruits is a very important activity, corresponding to 30% of total fruit production, with an economic importance of around 300 millions of Euros. Apple and pear are the most important, with an extension of 90,000 ha and a production of 1,734,000 t per year in the latest years, which represents 13.2% of total European production (MAPA, 1999). Spain is the second European country in the production of pear, and the fourth one in the production of apple. About 70% of total Spanish production of pome fruit is concentrated in the Ebro river valley and its influence zone, mainly Catalunya (Lleida), Aragon, and La Rioja. The most frequently planted apple cultivars are Golden Delicious, Starking and some autochthon cultivars of cider apple. In reference to pear trees, the major summer pear cultivars are Blanquilla, Dr Jules Guyot and Coscia.

The production of ornamental species is also a very important economic activity in Spain. Around 163 millions of ornamental plants were produced in 1998, with a field extension of 219 ha (MAPA, 1998).

3. HOST RANGE

E. amylovora is a pathogen that affects plants from the *Rosaceae* family and has a wide host range. Fire blight has been described in around 200 species included in 40 rosaceous genera (van der Zwet and Keil, 1979). Among the four subfamilies of the *Rosaceae, Maloideae* (syn. *Pomoideae*), *Rosoideae, Amygdaloideae* (syn. *Prunoideae*), and *Spireoideae* only in the last, fire blight has not been yet described. The most affected subfamily is the *Maloideae* although it has also been described in raspberry (*Rubus idaeus*) belonging to the *Rosoideae* subfamily (Starr *et al.,* 1951) and recently in Japanese plum (*Prunus salicina*) belonging to *Amygdaloideae* subfamily (Mohan and Thomson, 1996) (Table 2).

It is likely that fire blight is restricted to members of the rosaceous because sorbitol, a sugar alcohol found mainly in the *Rosaceae* family, could be a prerequisite for *E. amylovora* to colonize plants (Plouvier, 1963). It has been described that rosaceous plants use not only sucrose but also sorbitol for the transport and storage of carbohydrates (Wallaart, 1980). Sorbitol is the main transport sugar in apple and pear. Disruption of the sorbitol uptake, *srl* operon, in *E. amylovora,* resulted in loss of pathogenicity in apple seedlings (Aldridge *et al.,* 1997). Moreover, sorbitol is a good carbon source to synthesize and to increase exopolysaccharide (amylovoran) synthesis in *E. amylovora* (Bellemann *et al.,* 1994).

Table 2. Rosaceous subfamilies, genera and species in which fire blight has been described (van der Zwet and Beer, 1995)

Subfamily	Fruit trees	Ornamentals
Maloideae	*Eriobotrya japonica* (Loquat) *Cydonia oblonga* (Quince) *Malus domestica=Pyrus malus* (Apple) *Mespilus germanica* (Medlar) *Pyrus communis* (Pear) *Pyrus pyrifolia* (Asian pear)	*Amelanchier* spp. (Service berries) *Chaenomeles japonica (*Japanese quince*)* *Crataegus monogyna* (Hawthorn) *Crataegus azarolus* *Cotoneaster* spp. (Cotoneaster) *Pyracantha* spp. (Firethorn) *Sorbus aria* *Sorbus aucuparia* *Sorbus domestica* (Mountain ash) *Stranvaesia* spp. (=*Photinia* spp.)
Amygdaloideae	*Prunus salicina* (Japanese plum)	
Rosoideae		*Rubus idaeus* (Raspberry bush)

Although pear and apple are susceptible to fire blight, there are differences among the cultivars in their susceptibility to disease. The relative level of susceptibility to fire blight among the most common apple and pear cultivars and rootstocks is listed in Table 3. Apple cultivars Idared, Jonathan, Reine des Reinettes, Rome Beauty and Braeburn are extremely susceptible, while 'Fuji', 'Gala', 'Golden Delicious' 'Granny Smith', and 'Jonagold', are moderately susceptible and 'Golden Spur' is slightly susceptible. Among cider apple the most susceptible cultivars is Avrolles; 'Peau de Chien' is moderately susceptible, and 'Judor' is low susceptible. Most frequently planted pear

cultivars are highly susceptible to fire blight as Abate Fetel, Bartlett, Doyenne du Comice, General Leclerc, and Passe Crassane, or moderately susceptible as 'Guyot'. Cultivars Blanquilla, Conference, Coscia, Harrow Sweet, Maxine and Magness and rootstocks Old Home X Farmingdale as well as *Pyrus betulifolia* and *P. calleryana* show low susceptibility to the disease.

Table 3. Susceptibility to fire blight of commercial cultivars or species of apple, pear and rootstocks (van der Zwet and Beer, 1991; Le Lézec *et al.,* 1997)

High	Moderate	Low
Apple		
Idared	Fuji	Golden Spur
Jonathan	Gala	
Reine des Reinettes	Golden Delicious	
Rome Beauty	Granny Smith	
Braeburn	Jonagold	
Cider apple		
Avrolles	Peau de Chien	Judor
Apple rootstocks		
M.9	M.7	Novole
M.26		Robusta 5
Pear		
Abate Fetel	Docteur Jules Guyot	Blanquilla
Bartlett		Conference
Doyenne du Comice		Coscia
General Leclerc		Harrow Sweet
Passe Crassane		Maxine
		Magness
Pear rootstocks		
Bartlett seedlings	Quince (*Cydonia oblonga*)	Old Home X Farmingdale
Winter Nellis seedlings		*Pyrus betulifolia*
		P. calleryana

It seems reasonable not to discard the possibility that fire blight could affect new plant species, since European plum (*P. domestica*), peach (*P. persica*), nectarine (*P. persica* var. *nucipersica*) and sweet cherry (*P. avium*) cultivars, and almond (*P. dulcis*) have shown very limited resistant reaction to the plum isolate of *E. amylovora* under greenhouse conditions (Mohan and Bijman, 1999). These authors suggest that *Prunus* species are potentially susceptible hosts to fire blight that may act as reservoir for the pathogen.

On the other hand, it seems that strains of *E. amylovora* are not host species-specific. Most strains of *E. amylovora* isolated from apple are also pathogenic on pear, on other species of the *Maloideae* and on *Prunus* (Mohan and Thomson, 1996). However, strains of *E. amylovora* isolated from *Rubus* species are not pathogenic on pear or apple (Starr *et al.,* 1951; Heimann and Worf, 1985).

4. PATHOGEN AND PATHOGENESIS

Erwinia amylovora is a Gram-negative plant pathogenic bacterium, belonging to Proteobacteria division, γ subdivision and the family of *Enterobacteriaceae*. It is rod shaped (3 μm long by 0.5-1 μm in diameter) and moves by peritrichous flagella. *E. amylovora* is a facultative anaerobe.

E. amylovora is unable to reduce nitrate to nitrite. This characteristic separates *E. amylovora* from other species belonging to the genus *Erwinia* which are able to reduce nitrate to nitrite. Other cultural characteristics are that this species only ferments a small number of carbohydrates and nicotinic acid is required for its growth (Starr and Mandel, 1950; Lelliott and Dickey, 1984). The nicotinic acid is not a growth factor common among the genus *Erwinia*, and it has been included in the biochemical profile test for *E. amylovora* characterization. The mol% G+C of the DNA of seven strains ranges from 53.6 to 54.1 (Lelliott and Dickey, 1984).

The first studies on the physiological characteristics of *E. amylovora* showed that the rate of growth and the generation time vary considerably with the nutrients of medium and the incubation temperature. The *in vitro* generation time of *E. amylovora* is around 80 min at 30°C (Hildebrand, 1954). Although *E. amylovora* is able to growth between 3-5 ºC and at 37 ºC, the optimal temperature for its growth is 25-27 ºC (Billing *et al.,* 1961). The relationship between temperature and growth *in vitro,* was established by Billing (1974). She showed that from 9 ºC to 18 ºC there was a linear relationship between doubling rate and temperature. A sharp change was noticed in the growth rate at 18 ºC: an increase of 10 ºC from 18 ºC induced a moderate decrease in doubling time (from 2.1 h to 1.3 h), while a decrease of 10 ºC from 18 ºC induced a high increase of doubling time (from 14 h to 21 h). Therefore this value of 18 ºC (the turning point) is of special interest in the epidemiology of the disease.

PATHOGENICITY

Although fire blight is a necrotic disease, *E. amylovora* is a bacterium that does not produce plant cell wall degrading enzymes as virulence factors, like other species belonging to the genus *Erwinia*. Unlike many other plant pathogenic bacteria, *E. amylovora* does not secrete pectinolytic enzymes or phytotoxic metabolites that degrade cell wall polysaccharide (Seemüller and Beer, 1976). To infect its hosts, *E. amylovora* uses various factors including: extracellular polysaccharides, hrp/dsp gene products, and siderophores. Conversely to a number of plant-pathogen interactions, no specific *R/Avr* gene combinations have been described in the case of fire blight (Venisse *et al*., 2002).

In the last decades, with the use of powerful molecular tools, some pathogenesis related factors were found in *E. amylovora*. Molecular genetic studies of the bacterium have allowed the identification of various genes involved on its pathogenicity. Major known pathogenicity factors of *E. amylovora* are:

a. Exopolysaccharides (EPS). *E. amylovora* produces two EPS, amylovoran and levan, but only amylovoran (codified by genes *ams* 17 kb) is related to pathogenicity. Bellemann and

Geider (1992) deleted genes *ams* (for *am*ylovoran *s*ynthesis) and bacterium lost its virulence. Although amylovoran is needed for the virulence, this is not sufficient for the development of fire blight symptoms (Bernhard *et al.,* 1996). It seems evident that amylovoran may protect the bacteria against the host defense reactions, but the precise role for EPS in the disease process has not been established (Bugert and Geider, 1995). Genetic and environmental factors influence the expression of the *ams* operon. However, apparently *E. amylovora* does not regulate amylovoran production according to the growth stage (Bugert *et al.,* 1996).

b. Proteic factors codified by the *hrp-dsp* gene region (40 kb). The *hrp* region (for *h*ypersensitive *r*eaction and *p*athogenicity) controls the ability of bacteria to cause disease on host plants, and hypersensitive reaction (HR) on non host or resistant plants (Barny *et al.,* 1990; Dellagi *et al.,* 1998). The *hrp* cluster has been identified in the chromosome of *E. amylovora* and encodes three products classified on the basis of their functions: **regulatory proteins** which control the expression of other *hrp* genes, **secretory proteins** which are components of the Hrp secretion apparatus (type III secretion pathway or Hrp pathway) common to several plant necrogenic bacteria (*Erwinia, Pseudomonas, Ralstonia, Xanthomonas*) and animal and human pathogens (*Yersinia* and *Shigella*) (for review Hueck, 1998), and **secreted proteins** called effectors including harpins and potential effector's proteins (Kim and Beer, 2000). *E. amylovora* pathogenicity depends on a functional Hrp type III secretion system, involved in the secretion of pathogenicity proteins such as HrpN, HrpW, and DspA. Harpin, the product of *hrpN* gene is an extracellular elicitor of the HR (Wei *et al.,* 1992). The *dsp* (for *d*isease *sp*ecific) genes (*dsp*A and *dsp*B) are required for pathogenicity but dispensable for HR elicitation (Bellemann and Geider, 1992). While the *hrp* region is involved in electrolyte leakage induction in both interactions compatible and incompatible, the *dsp* region is essential for electrolyte leakage only in the compatible situation (Brisset and Paulin, 1992). Figure 2 summarizes most important events in the *E. amylovora*-plant cell interaction at the molecular level from genes and gene-product information currently known.

c. A siderophore (iron chelant) codified by the gene *dfo*. During plant tissue invasion process, low level of iron may be available for plant and pathogen, so host plant and pathogen compete for iron. Siderophores allow the pathogen to overcome conditions of iron limitation encountered in host tissues, and may also act as protective agents against iron toxicity. Recent works using mutants for one siderophore of *E. amylovora* (desferrioxamine=Dfo), have shown that the siderophore is implied in the pathogenicity of *E. amylovora* by two pathways: reducing the iron availability, and protecting bacteria from reactive oxygen species produced through the oxidative burst at the onset of infection (Dellagi *et al.,* 1998).

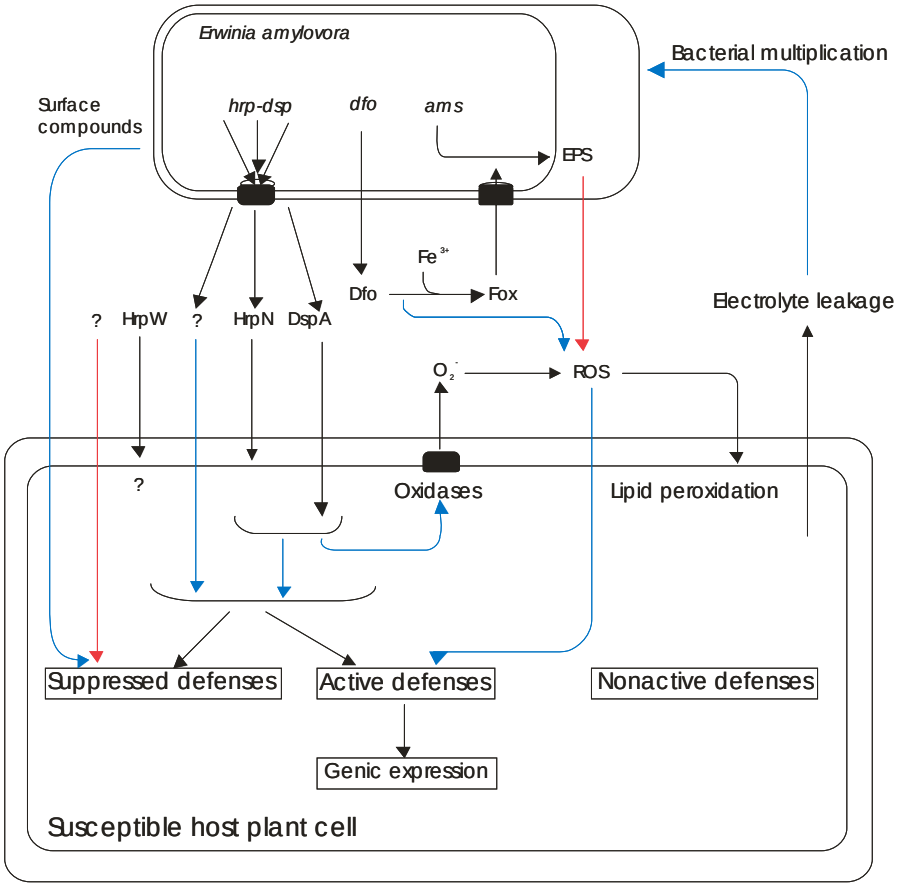

Figure 2. Molecular model for *E. amylovora*-host plant cell interaction. → negative effectors (inhibitors) → positive effectors (activators). Exopolysaccharide (EPS) controlled by the *ams* cluster; HrpW, HrpN and DspA are effectors secreted through the Hrp type III system. Dfo is a siderophore called desferrioxamine. ROS: reactive oxygen species (from Venisse *et al*., 2002).

5. SYMPTOMS AND DIAGNOSIS

The name ‘fire blight’ is descriptive of the most typical symptom of disease: a blackening of twigs, flowers and foliage as though they had been swept by fire. Depending on the plant part affected, many names such as blossom, twig, fruit, trunk and collar blight are frequently used (van der Zwet and Keil, 1979).

The first symptom of the disease appears in flowers. Flowers and young shoots are the most susceptible organs of the plant. Infection occurs through natural openings such as stomata, trichomes, hydathodes in leaves or shoots, and nectaries in flowers, or more frequently through wounds caused by hail, wind or human cultural practices. Fire blight is a systemic disease. Bacteria move through the intercellular spaces of parenchyma and in a later stage in the xylem vessels, provoking extensive lesions (Goodman and White, 1981). The disease spreads rapidly with a necrotic progression, and bacterium invades entire cluster, or progress into the twig. With warm humid weather or during wet conditions, ooze droplets sometimes exude from flower peduncle or blighted shoots (Figure 3.A). The bacterial ooze contains mainly bacterial polysaccharides and plant sorbitol (Eden-Green and Knee, 1974). Infected **blossoms** first appear water-soaked, then wilt, turn brownish to black and die. Infected blossoms may fall or remain attached to the tree, being a useful symptom in detecting blighted trees from distance. Infected **shoots** turn dark brown to black in pear, and red to dark brown in apple. Infection of growing shoots often results in characteristic shepherd’s-crook symptom which is accompanied or followed by a gray-black discoloration of the stem and attached leaves (Figures 3.B, C and D). Infected twigs shrivel; their tips curl over, and become dark brown or black colored. In few days, the infection can move 15-30 cm or more into the twig (van der Zwet and Keil, 1979). In **leaves** the infection progresses across the main veins, then into the petiole, and finally through the stem. Leaves turn brown in apple and black in pear. Blighted leaves also remain in the tree. **Young fruits or immature fruits** often become infected; they turn black, appear dried and shriveled, and usually remain attached to the tree, taking on a mummified appearance. Cankers appear as a result of necrotic lesions in the trunk of tree, and are the sites where the bacteria overwinter. Active fire blight cankers have a dark, water-soaked appearance (Figures 3.E and F).

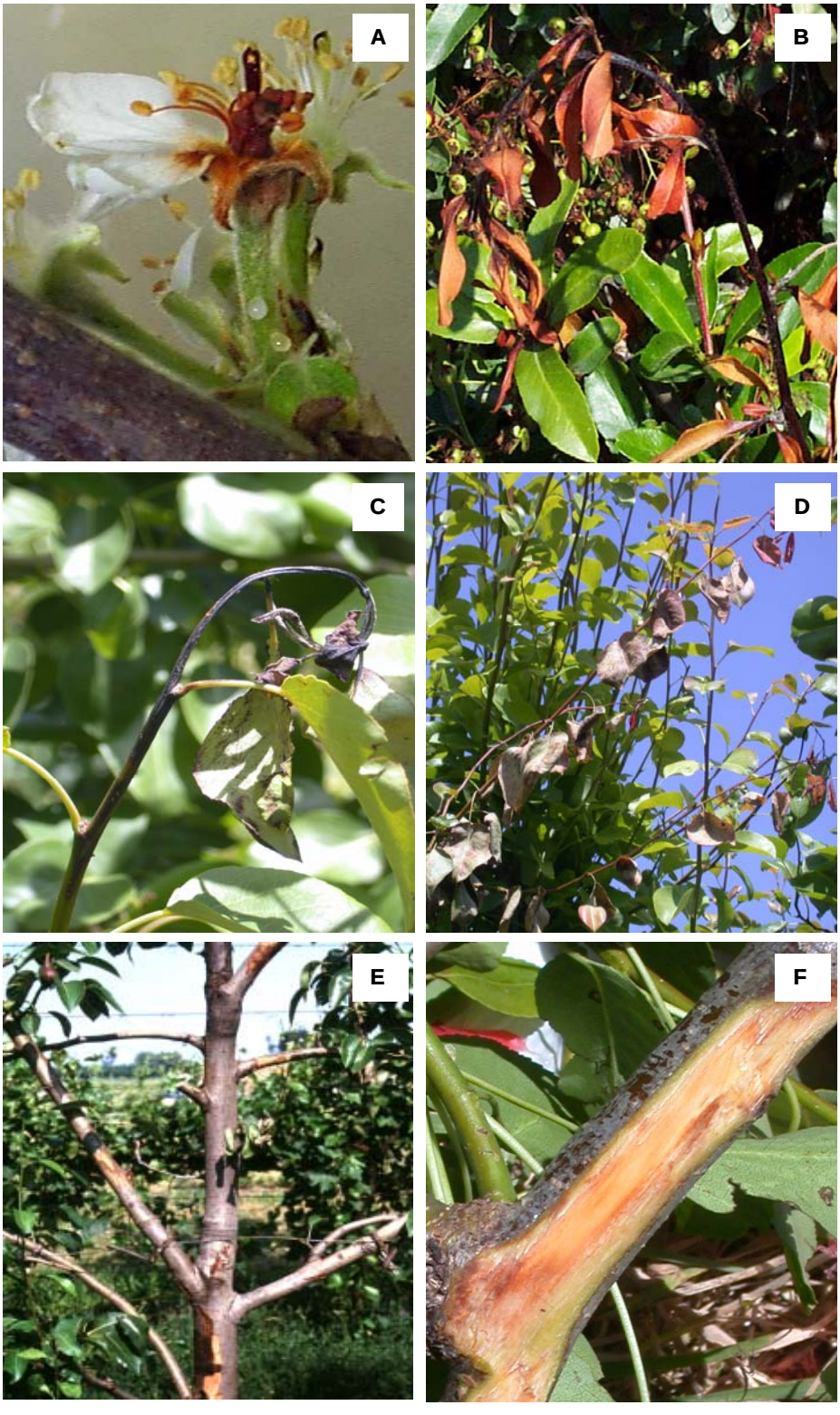

Figure 3. Fire blight symptoms. Production of exudates in apple flowers (A). Shoot infection on *Pyracantha* (B) and pear (C, D) showing wilting, the typical shepherd's crook. Cankers in pear trees (E, F).

DIAGNOSIS OF FIRE BLIGHT

In the early stages of infection, symptoms can be confused with those of other diseases. Among the diseases resembling fire blight there are blossom blast, caused by *Pseudomonas syringae* pv. *syringae*, although the absence of ooze in blossom blast is one of the features used to separate both diseases, or Nectria twig blight (coral spot), caused by the fungus *Nectria cinnabarina* (anamorph: *Tubercularia vulgaris*). The fungal disease can be distinguished by the characteristic bright-orange fungal fruiting structures on the canker surface (van der Zwet and Keil, 1979). Additionally, the distinguishable symptoms of fire blight are not always clearly observed, and it is difficult to diagnose the disease, so methods for *E. amylovora* identification are needed. Selective and differential media, such as CCT (Ishimaru and Klos, 1984), pathogenicity tests on pear or apple seedlings, molecular techniques such as PCR (Bereswill *et al.,* 1992), and ELISA or immunofluorescent techniques (Gorris *et al.,* 1996) can be used to isolate and identify the pathogen. Indeed, unambiguous, specific and sensitive methods for detecting *E. amylovora* in nursery stock are needed to prevent the introduction of the pathogen into fire blight-free regions and to limit and control the spread of new strains among regions where fire blight currently exists.

6. DISEASE CYCLE

Probably the most important step in the disease cycle is the overwintering of the bacteria, surviving previous year in plant infected tissues. It is well documented that *E. amylovora* may live for long periods of time or overwinter in the bark without producing blight symptoms (van der Zwet, 1969). It has also been demonstrated that *E. amylovora* can be isolated from buds, shoots, flowers and xylem vessels of symptomless plants. As weather becomes warm in the spring, the bacteria multiply in plant tissues and ooze are exuded to the plant surfaces in sticky droplets, and are dispersed to new flowers by insects or rain. Once on the flower stigmas (sticky pollen receptors), bacteria multiply rapidly, and are easily moved from flower to flower by bees. Bacteria on the stigmas can build to very high levels during warm bloom periods, but infection does not usually occur unless they are washed by rain to natural openings (nectaries) at the flower base. Bacteria can migrate from one infected flower to the rootstock, killing the tree in a season (Vanneste, 2000). During spring and summer various infection periods can take place. In autumn cankers appear in the branches where bacteria remind endophytous until the next vegetative period (Figure 4).

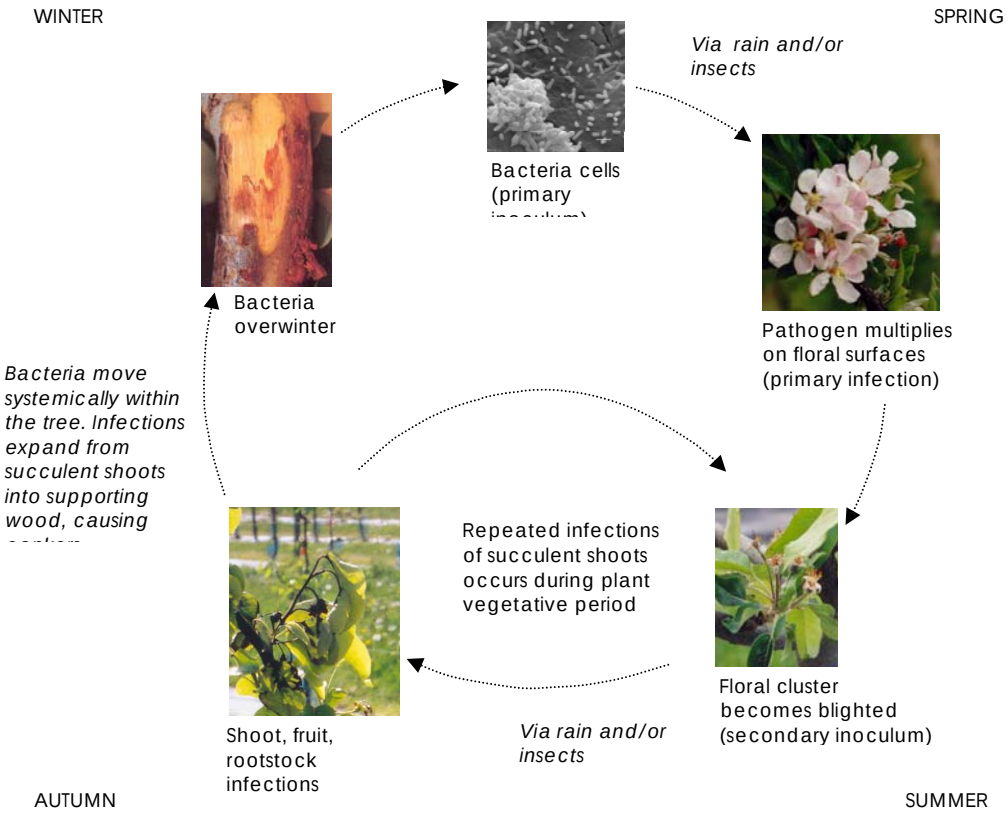

Figure 4. Illustrated disease cycle of fire blight.

7. EPIDEMIOLOGY

Like all plant diseases, fire blight needs three factors that simultaneously interact for disease development:

a. **Pathogen** (*E. amylovora*). In regions where the disease is established the presence of inoculum is assured by infected plant material. In disease-free regions, the presence of inoculum depends on the level of spread. Indeed, insects, wind and rain are believed to be primarily responsible for transmission in small areas. More often, pollinating insects attracted to the sweet smelling ooze are responsible for transmitting the bacteria that survive in ooze (ooze contains sugars that avoid dryness and bacteria), whereas birds (Seidel *et al.,* 1994), contaminated wood and the sale of infected plants, may disseminate the bacteria over large distances and can lead to

wide disease range distribution. The amount of inoculum is also a determinant factor related with the intensity of the disease, although few bacterial cells are enough for infection. In 1972, Crosse and colleagues demonstrated that median infective dose of *E*. *amylovora* (ID_{50}) in susceptible plant parts was around 100-1000 viable bacteria per milliliter.

b. **Susceptible host**. The susceptibility of a particular plant varies by cultivar, phenological stage, age, and the cultural practices employed on its culture. However, only some rosaceous species are susceptible to disease, and within that, some cultivars are more susceptible than others, as reported in Table 3. Moreover, the most susceptible vegetative periods of host to disease are blossom, and intense growth. To prevent succulent tissues formation and over-production of shoots, irrigation and fertilization should be carefully regulated. Nutritional stage (too much vigor) and edaphological factors (bad-drained soils) also increase the severity of disease.

c. **Optimal weather conditions**. Favorable weather conditions are determinant for disease development, mainly temperature and moisture, which affect both, host and pathogen, especially the growth of the pathogen. Temperatures of 25 ºC and relative humidity around 80% allow quick bacterial multiplication. The same environmental conditions also influence vector activity and thus affect the dissemination and the inoculation stages.

8. CONTROL

Control of fire blight is based on measures to avoid the spread of pathogen, and the introduction of disease in non-endemic regions. In European countries where fire blight has not yet been observed or described, the most important control measure is to make all possible attempts to keep the disease out of the country (van der Zwet and Keil, 1979). In these countries, a strict quarantine is necessary to prevent the import of plant material that may harbor the bacteria. Such regulations should not preclude the importation of fire blight hosts, but prevision should be made for strict inspection, testing, and determination that the material is free of *E. amylovora*.

In areas where the disease is described and causes severe losses, control of fire blight is also preventive and based on the knowledge on epidemiology and pathogenicity. Although nowadays more aspects of the disease are known since it was first described, there are still many facets unknown, such as molecular and genetic factors of host-pathogen interactions, this difficult the development of new control methods of disease. This knowledge would allow a better use of disease control systems.

Once the disease is introduced in one region only integrated methods are effective, and a prolonged control program is needed to minimize losses from the disease. An integrated approach combines: (a) practices that minimize host susceptibility and disease spread (regulatory, cultural

practices and breeding for resistance); (b) reduction of the amount of inoculum in the orchard (physical, chemical and biological control); and (c) well-timed sprays of bactericides to protect against infection under specific sets of conditions (risk assessment systems).

8.1. Regulatory measures

Regulatory control measures aim at excluding a pathogen from a host or from a certain geographic area (Agrios, 1997). To prevent the import and spread of pathogens into areas where they are absent, the laws regulate the conditions under which certain crops may be grown and distributed. Regulatory control is applied by means of quarantines, inspections or eradication of certain host plants. The European Community published in 1997 the directive 97/3/CE of the Council, modifying the previous directive 77/93/CE, related to the protection measures against the introduction into the Community of harmful organisms to the plants or the crop products and against their propagation in the Community. Each country in the EC established one net for supervise, control and keep free fire blight-areas or countries, and all susceptible plant material was commercialized under the Phytosanitary Passport. Particular areas within the EC have been designated as Protected Zone against certain pests and diseases which are either widely established elsewhere in the Community or restricted to special crops which are of only limited economic interest to the whole Community. Particular plants sent into these zones which can be host to the relevant pest or disease must be accompanied by a plant passport which includes a special code indicated by the letters ‘ZP’ (for Protected Zone). This code indicates that plants accomplish the EC requirements in reference to the quarantine pest or disease and may enter in Protected Zone.

Spain is a Protected Zone for fire blight, and all plant material is marketed with label ZP-E (for Spain) or ZP-b2 (for all protected zones of fire blight in the Community). In Spain different laws have been published that culminates with the RD (Royal Decree) 1201/1999 by which Spanish government has established the National Program of Eradication and Fire Blight Control of the Rosaceous (BOE, 1999).

8.2. Cultural practices

The most effective cultural practices in fire blight control are the avoidance of susceptible cultivars and rootstocks in new plantings. Resistant or low tolerant varieties of apple and pear should be planted wherever possible. Horticultural practices that help to reduce the severity of disease include planting new orchards on well-drained soils and promote early cessation of growth without excessive reduction of tree vigor (such as applying nitrogen fertilizer early and cultivating no later than in midsummer). Another cultural practice that will help to control fire blight is carefully pruning of trees during dormancy to remove overwintering cankers.

8.3. Breeding for resistance

There is not any cultivar of pear, apple or rootstock totally resistant to fire blight. It is due to the lack of only one gene involved in the resistance as well as the complex inheritance (Malnoy, 2001). In

spite of that, various breeding programs have been developed in Europe and USA. The aim of the apple and pear breeding programs is the combination of different sources of resistance and good fruit quality in new cultivars. Genetic resistances are more often identified in primitive species or forms, or in obsolete cultivars with rather poor appearance and quality (Lespinasse and Aldwinckle, 2000). Research is on-going in breeding for fire blight resistance using traditional breeding techniques and new biotechnological methods, as genetic engineering.

8.4. Biological control

Biological control is the use of natural or modified organisms, genes, or gene products to reduce the effects of undesirable organisms (pests), and to favor desirable organisms such as crops, trees, animals, and beneficial insects and microorganisms, according to the definition proposed by the National Academy of Sciences (NAS, 1987 in Thomashow and Weller, 1996). A more recent and simplest definition of biological control is 'the total or partial destruction of pathogen populations by other organisms' (Agrios, 1997). According to Sutton and Peng (1993) biological control methods and strategies involve the timely manipulation of antagonist populations to suppress pathogens in various inoculum sources or on host plants. In agriculture, biological antagonisms, although subject to numerous ecological limitations, are expected to become an important part of the control measures against diseases. The use of biocontrol agents for biological control is one of the most promising alternatives to chemical control, either alone or as part of an integrated system to reduce pesticide inputs.

The mechanisms by which antagonistic microorganisms affect pathogen populations are not always evident, but they are generally attributed to one or more effects as:

1. Predation or parasitism, interaction that results in the death of the pathogen.
2. Competition, exclusion of the pathogen due to nutrients and space competence.
3. Antibiosis, inhibiton of the pathogen growth by means of antibiotic substances released by the biocontrol agent.
4. Induced resistance in the host plant, the biocontrol agent triggers the resistant response of plant.

Biological control is achieved by means of the previous inoculation of epiphytic bacteria that compete or antagonise *E. amylovora*. In the USA, the strain A506 of *Pseudomonas fluorescens* is commercialized as Blightban® (Plant Health Technologies, ID, USA) for fire blight control and strain C-9.1 of *Erwinia herbicola* is under marketing process. The main advantages of biocontrol agents are that they help to reduce the use of chemical-based compounds and the risk of developing pathogen resistance to traditional chemicals, they are safer to use in most cases and they may be compatible with chemicals. However, the main disadvantages of biocontrol agents are that they tend to be more difficult to implement than chemicals and, in most cases, they are more expensive and infecttive than chemicals.

In order to finalize this section it is interesting to remark that not only non-phytopathogenic strains are studied for biological control of disease, but also avirulent mutants of pathogen have been

tested. Tharaud *et al.* (1997) obtained satisfactory results in the control of *E. amylovora* infection with avirulent mutants of the bacterium under laboratory conditions.

8.5. Physical control

Physical control methods are based on the use of factors as temperature or radiation in order to reduce or eliminate the inoculum. In fire blight control it is possible to use physical methods to avoid the disease spread and to reduce the amount of inoculum. Plants normally grow at a temperature ranging from 1 to 40 ºC, and most plant species grow better between 15 and 30 ºC. Perennial plants and dormant organs (e.g. seeds and corms) of annual plants may survive temperatures below or above the normal range (Agrios, 1997). The efficacy of heat therapy methods is based on the fact that dormant plant material can support high temperatures at which the pathogen does not survive. The resistance of *E. amylovora* cells to high temperature was studied in an attempt to propose a technique for obtaining plant material free of internal contaminants by heat treatment (Aldwinckle and Gustafson, 1993; Keck *et al.,* 1995). It was found that temperatures of 45 ºC for 70 min or 50 ºC for 50 min were enough to destroy pure culture of the bacteria with some variations between strains tested (Keck *et al.,* 1995).

The solarization is another physical method that consists of covering a tree or a plant with a plastic film to increase the temperature. The inactivation or reduction of pathogen inoculum depends on the species, the environmental temperature and the year. In many solarized trees for the control of the fire blight, cankers expansion was stopped and no viable *E. amylovora* were isolated from cankers, while cankers on untreated trees continued expanding. Several consecutive days of temperatures near 33 ºC in 1994, raised temperatures inside the tents of 56 ºC, resulting in a complete eradication of the pathogen and in the death of tops of the trees (Thomson, 1996).

8.6. Chemical control

Chemical methods aim at protecting the plant from the pathogen inoculum that has arrived, or is likely to arrive, or curing an infection that is already in progress. Some more recent, still experimental chemicals operate by activating the defenses of the plant (systemic acquired resistance) against pathogens (Agrios, 1997).

Chemical treatments are very useful and play an important role in the effort to eliminate losses in fruit production due to infection by *E. amylovora* (Psadillas and Tsiantos, 2000). However, chemical control of fire blight is very slightly effective in affected plants, for which it is only possible to use it to preventively avoid the diffusion from the first focus. Among chemical compounds, only the antibiotics (streptomycin, oxytetracycline, kasugamycin and flumequine) and copper derivatives (copper sulfate, copper oxychloride, and copper hydroxide) have shown certain efficacy in the control of the disease.

Other products act at the interaction host-pathogen level, modifying the physiology of the host or inducing the systemic resistance. Some of them show direct low antimicrobial activity, while others do not (de Waard *et al.,* 1993). Fosetyl-Al and ethephon belong to the first group, whereas

dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) are included in the second group since they do not present direct antimicrobial activity and act on the system of defense of the plants (Guest, 1984; Lawton *et al.,* 1996).

Some authors suggest that the prevention of blossom infection by *E. amylovora* is the key to fire blight management (Thomson *et al*., 1982; Johnson and Stockwell, 1998). Bactericide sprays may prevent blossom infections but blossoms that open after spraying are not protected. Consequently, for optimal chemical protection, sprays should be applied frequently with short intervals between them to protect newly opened blossoms. This is not practical due to the cost of spraying and the environmental considerations. The solution is to apply the bactericides only when needed according to a warning system (Shtienberg *et al.,* 2003).

8.7. Risk assessment systems

Risk assessment systems were developed to ensure optimal timing for applications and to guide prospections in relation to risk infection. An epidemiological model predicts the likelihood that the disease occurs in base of weather factor observations. As fire blight occurs so sporadically in time and space, various prediction models have been studied and proposed. Models identify favorable periods for the epiphyte growth of *E. amylovora* in flowers, before the event of infection happens. They are extensively used to determine the need and the optimal moment for chemical sprays.

From fifty's to now several forecast systems have been developed in USA and Europe. The first models were developed in USA by Mills (1955) and Powell (1965) on the basis of the effect of temperature on the apple and pear blossom blight. Later in Europe Billing developed and improved Billing's systems (Billing's original system in 1980; Billing's revised system in 1990 and Billing's integrated system in 1996) based on the effect of temperature on the multiplication of *E. amylovora* and the bacterial population needed for infections to occur. The forecast systems evaluate weather patterns for the risk of fire blight. The Billing's systems have been widely used to assess past weather conditions and to predict the likelihood of disease occurrence in areas where the pathogen does not yet exist. At the moment, a computer program developed in USA, the Maryblyt model (Steiner, 1990), is available for growers to keep track of fire blight infection periods for bactericide application and to predict when symptoms will occur.

9. STRATEGIES FOR THE MANAGEMENT OF FIRE BLIGHT IN SPAIN

As it has been described previously, fire blight was first described in Spain in 1995, in the Northeast of the country. From the first outbreak, regulatory methods such as inspection to identify potentially infected plants and an eradication policy was rapidly implemented to control new introductions and the spread of the pathogen in Spain. In the last years, new outbreaks of the disease appeared located to the same reduced geographical areas. In most cases there were

difficulties to establish the origin of fire blight introduction in non-endemic protected areas. The hypothesis of an introduction of plant material harboring latent infection of *E. amylovora* was claimed on the basis of previous evidences; however this was never proved for the Spanish outbreaks (Calzolari *et al.*, 1982). In regions where fire blight has still not been detected, as Catalunya, continuous prospections must be done and methods for sanitation of plant propagation material of susceptible hosts must be developed. In areas where the fire blight has been introduced, eradication and protective control methods must be implemented (including cultural practices and chemical sprays) in order to reduce the amount of inoculum.

The incidence of the disease is still low in fruit-producing areas; however, taking into account the rapid progression of the disease in other Mediterranean countries, it is necessary to establish appropriate management strategies to minimize the potential losses.

Phytosanitary measures on a legislative basis have been applied in collaboration with the Ministry of Agriculture and regional authorities in order to prevent further damage. Prevention of fire blight will remain the most important task in the near future and needs intensive cooperation of all the parties concerned, as fruit growers, legal authorities, scientific community and environmentalists.

Specific cultural practices for fire blight eradication and prevention are recommended and generally used in Spain, mainly in regions where pome fruit production is important and the potential infection risk is high. Nevertheless, ignorance of the disease by growers, economical interests or a point of view too much practical can lead to avoid these practices and therefore to the appearance and development of the disease.

In order to avoid the introduction and spread of the pathogen by means of symptomless plant propagation material in disease-free regions, it is necessary to develop a method that allow to reduce or completely eliminate the pathogen before the use of this plant material in nurseries or orchards. The heat treatment (thermotherapy) could be effective. However, there is very little information available on heat treatment of rosaceous plants to eradicate *E. amylovora* from propagation material. Studies performed by Keck *et al.* (1995) in Austria showed very promising results. In fire blight-free zones of Spain, as Catalunya, the plant material that enter must be warranted in order to avoid the pathogen introduction in symptomless samples, therefore it is necessary to determine which heat treatments can be made and their effect on the survival of the plant material.

About chemical control indeed only chemicals like copper compounds and antibiotics are used in countries affected by fire blight. However, there are many problems associated with their use such as:

1. Selection for resistance in pathogen since it reduces its effectiveness. In *E. amylovora* resistance to streptomycin is well documented while there is little information about resistance to copper.
2. Restricted use of compounds in some countries. For example, the streptomycin can not be used in Spain where only the kasugamycin is allowed.
3. Limited action on the plant surface.
4. Phytotoxic effects not desired in host.
5. Environmental contamination for the generation of residues.
6. Effects on human and animal health.

Conventional chemical products used for fire blight control have great problems of use and it is necessary the evaluation of new products. Some alternative products act by means of stimulation of plant defense mechanisms known as systemic acquired resistance (SAR). This group includes the acibenzolar-S-methyl ester (ASM) (Bion® or Actigard®) which is a benzothiadiazole chemically similar to salicylic acid, and harpin (Messenger®) a new product that also seems to induce the SAR. Harpin is the product of *hrpN* gene of *Erwinia amylovora* and elicits hypersensitive response and disease resistance in many plants. The commercial product has been more effective in annual than in perennial plants. These new products could be evaluated for preventive control of fire blight. From this point of view it would be interesting to investigate if they are effective in fire blight control, and which is their activity in *E. amylovora*-host interaction. Recently, prohexadione calcium (Apogee®, registered in Spain as Regalis®), a growth regulator, has been effective in the management of shoot blight infection, in experimental trials conducted in USA. Prohexadione calcium is ineffective for controlling the blossom blight phase of the disease, and is only registered for its use on apple. But at the moment, none of these products are commercially available in Spain for fire blight control.

Moreover, biological control can be a very useful tool for disease control but any product is still commercialized in Spain for biological control of fire blight. A limitation of biological control is the low effectiveness of these products under different environmental conditions. Several studies suggest that their efficacy decreases if weather conditions are not the optimal. Biological control of fire blight in Spain seems a long-term strategy. However, very promising results have been obtained by Plant Pathology Group of University of Girona (Cabrefiga, 2000).

Risk assessment systems can also be a very useful tool to guide prospections and chemical sprays in Spain. Indeed, Montesinos and Llorente (1999) elaborated a preliminary fire blight risk map for Northeastern country according to previously described fire blight models.

Finally, the development of resistant cultivars is the long-term approach for fire blight control, and at the moment in Spain a cultivar conversion is not yet needed, although the most currently used cultivars are susceptible.

10. NEW PERSPECTIVES IN FIRE BLIGHT CONTROL

Changes in modern orchard management practices and market demand over the two last decades have pre-empted the widespread use of resistance and reduced fertility as options for lessening the risks of fire blight (Steiner, 2000).

Due to the low effectiveness of the currently available methods for fire blight control, already commented, and to the present situation of the fire blight in Spain it is necessary to carry out studies that allow to know and to determine the best strategies for preventing and controlling the disease in Spain. In order to prevent the entrance of fire blight pathogen in disease-free regions, it

is necessary to develop strategies based on thermotherapy as a quarantine or prophylactic method. Moreover, as chemical control is currently the most effective control method in countries or regions where the disease is well established, the improvement and optimization of chemical control methods, and the evaluation of new compounds like benzothiadiazole and phosphonate derivatives in fire blight control, are unavoidable. Additionally, the knowledge of the effect of these compounds in *E. amylovora*-host plant interaction is of special interest to elucidate the mode of action of these products. Finally, in order to guide prospections and to support chemical control measures, available fire blight risk prediction models must be evaluated under Spanish weather conditions.

This work is framed in the research lines of Plant Pathology group of the Universitat de Girona, which research is focused in developing new strategies of plant disease control, in order to minimize the use of chemical pesticides.

OBJECTIVES

The main objectives of this work were:

1. To determine the efficacy of thermotherapy for the eradication of *Erwinia amylovora* from propagating plant material.

2. To evaluate and improve the effectiveness of chemicals with different mode of action (bactericides and plant defense activators) in control of fire blight. Additionally, in order to understand their mode of action, to study at cytological and molecular level their effect in *E. amylovora*-pear interaction.

3. To elaborate maps of fire blight infection risk, to establish the regions where the probability of fire blight risk is high, to guide the preventive inspections, and to determine the origin of outbreaks that had taken place in Spain.

CHAPTER 1

THERMOTHERAPY FOR ERADICATION OF *Erwinia amylovora* FROM PROPAGATING PLANT MATERIAL

1. INTRODUCTION

As has been commented in the general introduction, contamination of propagation plant material is considered as a source of potential risk for the dissemination of fire blight. As the pathogen is classified as a quarantine organism, phytosanitary restrictions for the transference of plants are established. Although plant material introduced into fire blight free areas (such as Catalunya) is certified with the ZP passport, symptomless plants can carry endophyte inoculum.
In order to eliminate potential inoculum of *E. amylovora* from symptomless rosaceous propagation material, heat treatment (thermotherapy) could be useful.

1.1. Thermotherapy

Thermotherapy consists of heat treatment of plant parts or dormant whole plants at a temperature-time regime that kills the conserved pathogen and that is only slightly injurious to the host plant (Grondeau and Samson, 1994). The temperature and the axposure time of the treatment change in accordance with the different combinations that are established between the host and the pathogen, so that they do not harm the host and that reduce or eliminate the pathogen populations. Treating plant material with heat is a-century-old method of disease control that has proved to be efficient against various pathogenic microorganisms (Jensen, 1887 and 1888 in review of Grondeau and Samson, 1994). Thermotherapy is frequently used along with meristem culture (Nyland and Goheen, 1969). Several earlier reviews referred to the use of heat therapy in control of virus diseases but, in the last twenty years, some authors have indicated that this method is useful for bacterial disease control, too (Table 1.1).

Table 1.1. Heat treatments effective in reducing or eliminating plant pathogenic bacteria from plant material

Pathogen	Plant material	Heat treatment	Reference
Agrobaterium tumefaciens	Dormant cuttings of grape	Hot water (50 ºC for 30 min)	Burr *et al.,* 1989
Clavibacter michiganensis subsp. *michiganensis*	Tomato seeds	Hot water (52-56 ºC for 20-30 min)	Fatmi *et al.,* 1991
Erwinia amylovora	Dormant buds of apple	Hot water (48 ºC for 60 min)	Aldwinckle and Gustafson, 1993
E. amylovora	Apple and pear shoots	Moist and dry heat (45 ºC for 5 h)	Keck *et al.,* 1995

As suggested by Geard (1958), the most plausible hypothesis for the mechanism of heat therapy is that high temperatures cause the destruction of essential chemical activities in both pathogen and host but the host is more able to overcome the damage produced by the heat therapy. In other words, the temperature coefficient of thermal inactivation for the host exceeds that of the pathogen at certain temperatures. According to Agrios (1997) the mechanisms by which high temperature injure plants are through inactivation of certain enzyme systems and acceleration of others, thus leading to abnormal biochemical reactions and cell death. Plant material in dormancy such as seeds, yolks, grafts, and tubers, is normally treated with warm water at temperatures between 35 and 54 ºC, with treatments that last from some minutes to several hours. Plants that present active growth are treated with warm air, which favors plant survival and eliminates the pathogen. Temperatures between 35 and 40 ºC seem to be optimal to treat the growing plants with warm air (Agrios, 1997).

Thermotherapy treatments include hot water, hot air, solar heat, aerated steam, and radiation. Heat is applied either by hot water in controlled-temperature tanks or by hot air in controlled-temperature chambers. Hot water was used earlier than hot air in plant heat treatments (Nyland and Coheen, 1969).

1.2. Applications

Heat therapy is a simple, effective, economical, and environmentally safe method for eradicating bacterial pathogens from dormant plant material. Heat treatment should be adopted as a standard procedure for the movement of plant through quarantines (Nyland and Goheen, 1969).

Although heat is an important therapeutic agent for plant diseases, plants in the heat chamber usually show temporary damage or abnormalities (Nyland and Coheen, 1969). Baker (1962) suggested that some of the causes of damage to plants or plant parts after hot water therapy are leaching, water-soaking, and asphyxiation of host tissues. Factors favoring survival of plants or plant parts under heat treatments are high carbohydrate reserve, maturity of tissues, partial dehydration that accompanies storage, previous growth at high temperature, and low humidity. However, the critical factors conferring temperature tolerance in higher plants are still poorly understood (Queitsch *et al*., 2000).

1.3. Use of thermotherapy in fire blight control

Fire blight has been reported throughout the apple and pear producing regions of North-America, Europe, Western-Asia, and New Zealand and it continues dispersing in other regions. Several studies have shown that *E. amylovora* is present in a low percentage of buds used for the propagation of rosaceous plants. Keil and van der Zwet (1972) indicated that *E. amylovora* could be isolated from 60% of the apparently healthy suckers that developed from blighted 'Bartlett' pear trees in the orchard. Calzolari *et al.* (1982) isolated *E. amylovora* from vegetative buds of 'Jonagold' apple imported into Italy from a Dutch nursery. These evidences suggest that fire blight can potentially be long-distance transported through propagation material from nursery. Not many reports establish the quantity of *E. amylovora* cells needed to cause an outbreak in a commercial fruit region, but it is assumed that very few cells are enough, since a small amount of inoculum is required to initiate an outbreak (Crosse *et al*., 1972). So, eradication of bacteria present in propagation plant material seems to be an effective method to control the dispersion of the disease. The effect of heat treatment has been evaluated by several authors for this purpose. However, this goal is not yet achieved for many crops, especially in the case of perennial fruit tree contaminations that frequently occur in nurseries.

A method that would eradicate *E. amylovora* from plant propagation material, maintaining shooting, would eliminate the risk of national and international transmission of *E. amylovora*. The spread of *E. amylovora* through propagation material from contaminated orchards originated different phytosanitary restrictions and expensive but often insufficient quarantine measures. Therefore, the elaboration of simple alternative procedures seems to be of importance. One of these methods could be direct heat treatment of scions in order to obtain healthy plant material. The effect of temperature on the growth rate of *E. amylovora* has been described (Billing, 1974). There has been success in eliminating *E. amylovora* from naturally infected plant material through thermotherapy (Keck *et al*., 1995). These preliminary results have been the basis of our work. Accurate studies must be performed in order to determine plant sensitivity to thermotherapy, since differences in susceptibility to heat damage may exist among varieties within plant species (Smoot and Segall, 1963). Additionally, it has been shown that both host and pathogen vary in their ability to tolerate heat treatments. Therefore, effective heat therapy conditions must be determined for each pathosystem.

OBJECTIVES

The aim of this work was to develop a method for eradicating viable cells of *Erwinia amylovora* from symptomless propagation wood of rosaceous species. The effect of heat type, temperature and exposure time of treatment on viability of dormant buds and on survival of *E. amylovora in vitro* and in plant material was determined.

2. MATERIALS AND METHODS

2.1. SENSITIVITY OF PLANT MATERIAL TO HEAT TREATMENT

2.1.1. Plant material

Sensitivity to heat treatment was evaluated on branches with dormant buds of different rosaceous species and cultivars, and on self-rooted micropropagated pear plants (Table 1.2). Detached branches (20-25 cm long, 6-8 buds) with leaf buds were taken from different commercial orchards and nurseries of Girona (Spain). The pear budwoods were collected in winter (phenological stage A) (Fleckinger, 1965). The ornamental budwoods were collected in late winter with buds at B phenological stage. All branches were introduced into plastic bags, and maintained at 1 ºC until heat treatment. Self-rooted plants of cv. Conference (CAV clone) obtained by micropropagation were also used. Plants were 2 to 3 years old, about 30 cm high and were grown in 1 L capacity plastic containers. Plants were chilled during winter and pruned in the greenhouse 2 to 3 months before experiments began. When the experiment was performed plants had dormant leaf buds.

Table 1.2. Species, cultivars and sources of rosaceous plants evaluated for their tolerance to heat treatment

Plant species	Cultivar	Source
Branches		
Pear (*Pyrus communis*)	Conference Abate Fetel Grand Champion	Can Ribes (Camós, Spain) Can Ribes (Camós, Spain) Can Ribes (Camós, Spain)
Cotoneaster dammeri	Skogholm	Vivers Tortadés (St. Hilari Sacalm, Spain)
Cotoneaster horizontalis		Vivers Multiplant (St. Gregori, Spain)
Cotoneaster wardii		Vivers Multiplant (St. Gregori, Spain)
Crataegus monogyna		Vivers Acycsa (Pont de Molins, Spain)
Sorbus aria		Vivers Acycsa (Pont de Molins, Spain)
Plants		
Pear (*Pyrus communis*)	Conference	Agromillora Catalana, SA (Subirats, Spain)

2.1.2. Heat treatment

Two kinds of heat treatment were tested, moist and dry heat, and two temperatures were applied in both, 45 ºC and 50 ºC, for different incubation times: 0, 3, 4, 5, 6, and 7 hours for 45 ºC and 0, 1, 2, and 3 hours for 50 ºC. Branches and plants were treated according to the two procedures described below.

Dry heat. Application of dry heat was performed in a waterbath (Hetofrig, Heto, Denmarck). Pear plants or branches were placed in sealed polyethylene bags, and introduced into the waterbath.

Roots of pear plants were wrapped with absorbent paper and introduced into polyethylene bags to avoid harmful effects on the root.

Moist heat. Pear plants or branches were wrapped with moistened absorbent paper, with one end of the paper dipped into distilled water (to ensure moist conditions), and maintained in an incubator (MLR 350H, Sanyo, Japan) at selected temperatures (45 ºC or 50 ºC). Roots of plants were protected as described above.

2.1.3. Assessment of heat sensitivity of branches and plants

In order to assess the tolerance of plant material to heat, treated branches were induced to sprout after heat application according to the procedure described by Montesinos and Vilardell (1991) after a modification of Szkolnik and Hickey (1986) method. Branches were placed in containers filled with 1 L of sterilized 1% sucrose solution, and forced to shoot in an environmental chamber at 22-25 ºC, 12 h light and 70-80% relative humidity. Periodically, the base end of branches was pruned and the sucrose solution was replaced to avoid yeast growth and occlusion of the plant vascular system. Under these conditions, the opening of buds occurred in about 2 or 3 weeks. Plants were placed in 1 L capacity plastic containers filled with commercial potting mix and were maintained in the greenhouse under natural photoperiod and fertilized once a week, for shooting induction. Percentage of sprouting was calculated as percentage of swollen shoot buds, one month after heat treatment.

2.1.4. Experimental design and statistical analysis

A completely randomized factorial design with four factors (cultivar, heat type, temperature, and exposition time) was used in branches, and three factors (heat type, temperature, and exposition time) in plants. Two heat treatments (dry and moist), two temperatures (45 ºC and 50 ºC) and different exposure times for each cultivar were evaluated. Each treatment was composed of 5 replicates. The experiment in plants was repeated twice. Non-heat-treated branches or plants were used as controls. The effect of each factor was determined by means of an analysis of covariance (ANCOVA) where exposure time was considered as the covariate, by using the general linear models (GLM) procedure of statistical analysis system (SAS) (Version 8.2, SAS Institute Inc., NC, USA). To test the homogeneity of variances the Bartlett test was used, and to evaluate the normal error distribution the Shapiro-Wilk test was used.

2.2. THERMOSENSITIVITY OF *ERWINIA AMYLOVORA* STRAINS

2.2.1. Bacterial strains and growth conditions

A total of 39 strains of *E. amylovora* from different sources were evaluated for their sensitivity to heat treatment (Table 1.3). Strains were selected for their different host plant and geographical origin. Among the strains, the PMV6076 avirulent mutant (*hrp*$^-$, *dsp*$^-$::MudIIPR13, Cm^r) of French strain CFBP1430 was included. Bacterial suspensions were prepared in sterile phosphate buffer (0.1 M potassium phosphate buffer at pH 7) from Luria-Bertani (LB) (Maniatis *et al*., 1982) agar cultures grown at 25 ºC for 24 h. Suspensions were overlayed with sterile paraffin oil to avoid buffer evaporation. The concentration of bacteria was adjusted to 10^8 cfu mL^{-1}. Concentration was determined using absorbance at 620 nm and interpolation in a previously obtained calibration curve.

2.2.2. Heat treatments and heat sensitivity assessment

Bacterial suspensions were immersed in a waterbath at 45 ºC or 50 ºC. These temperatures were chosen because they are lethal to many bacteria, whereas dormant buds can survive exposures of up to 150 min. Aliquots from bacterial suspensions were removed after 15, 30, 45, 60 and 80 min of exposure to 45 ºC, and after 10, 20, 30, 40 and 60 min to 50 ºC. The viability of recovered bacteria was assessed by plating serial 1:10 dilutions on Luria-Bertani (LB) agar and incubated at 25 ºC, for 48 h. After incubation time, *E. amylovora* colonies were counted. Colony growth was considered as tolerance to heat treatment.

2.2.3. Experimental design and statistical analysis

A completely randomized factorial design with 3 factors (strain, temperature, and exposure time) was performed, with 3 repetitions for each treatment. The data were analyzed using the GLM procedure of SAS (Version 8.2, SAS Institute Inc., NC, USA), and analysis of covariance was performed with the time variable considered as covariate. For all experiments, data were tested for the homogeneity of variances (Bartlett's test) and normal error distribution (Shapiro-Wilk test). Lethal thermal time for each strain and temperature was calculated by using a regression analysis (proc REG of SAS) with the natural log (Ln) transformed data of survival as colony forming units recovered each time divided by initial colony forming units (survival=cfu/cfu$_0$).

Table 1.3. Origin of strains of *E. amylovora* tested for their heat sensitivity

***E. amylovora* strain**[a]	**Host plant**	**Geographical origin**	**Year of isolation**
CFBP1430	*Crataegus* sp.	France	1972
CUCM273	*Malus pumila=M. sylvestris*	United States	1971
EPS101	*Pyrus communis*	Spain (Lleida)	1999
IVIA1614.2	*Pyracantha* sp.	Spain (Segovia)	1996
IVIAEa115	*Cydonia oblonga*	Bulgaria	1989
NCPPB311	*Pyrus communis*	Canada	1952
NCPPB595	*Pyrus communis*	United Kingdom	1958
NCPPB683	*Pyrus communis*	United Kingdom	1959
NCPPB1734	*Pyrus* sp.	Egypt	1965
NCPPB1819	*Crataegus* sp.	United States	1966
NCPPB2080	*Pyrus communis*	New Zealand	1953
NCPPB2291	*Rubus idaeus*	United States	1970
NCPPB2791	*Pyrus communis*	United States	1975
NCPPB3159	*Malus sylvestris*	The Netherlands	1980
NCPPB3548	*Eriobotrya japonica*	Turkey	1987
OMPBO1185	*Pyrus communis*	Italy	1998
PMV6076[b]	*Crataegus* sp.	France	1990
UPN500	*Pyrus communis*	Spain (Navarra)	1998
UPN506	*Malus sylvestris*	Spain (Gipuzkoa)	1998
UPN513	*Pyrus communis*	Spain (Gipuzkoa)	1998
UPN514	*Pyrus communis* cv. D. Comice	Spain (Gipuzkoa)	1998
UPN524	*Malus sylvestris*	Spain (Gipuzkoa)	1998
UPN529	*Pyracantha* sp.	Spain (Navarra)	1997
UPN530	*Pyrus communis*	Spain (Navarra)	1997
UPN536	*Malus* sp.	Spain (Guipuzcoa)	1998
UPN544	*Crataegus* sp.	Spain (Navarra)	1998
UPN546	*Cydonia* sp.	Spain (Navarra)	1998
UPN575	*Malus* sp.	Spain (Navarra)	1998
UPN576	*Pyracantha* sp.	Spain (Navarra)	1995
UPN588	*Pyracantha* sp.	Spain (Navarra)	1998
UPN609	*Sorbus* sp.	Spain (Navarra)	1998
UPN610	*Pyracantha* sp.	Spain (Navarra)	1998
UPN611	*Cotoneaster* sp.	Spain (Navarra)	1998
UPNEaZ7	*Pyrus communis*	Spain (Zaragoza)	2000
UPNEaZ9	*Pyrus communis*	Spain (Zaragoza)	2000
UPNEaZ13	*Pyrus communis*	Spain (Zaragoza)	2000
USV1000	*Pyrus communis*	Spain (Lleida)	1999
USV1043	*Pyrus communis*	Spain (Lleida)	1999
USV2194	*Pyrus communis*	Spain (Lleida)	1999

[a] CFBP: Collection Française de Bactéries Phytopathogènes, INRA, Angers (France); CUCM: Cornell University Collection of Microorganisms, Ithaca (USA); EPS: Escola Politècnica Superior, Universitat de Girona, Girona (Spain); IVIA: Instituto Valenciano de Investigaciones Agrarias, Valencia (Spain); NCPPB: National Collection of Plant Pathogenic Bacteria,York (United Kingdom); OMP-BO: Osservatorio per le Malattie delle Piante, Bologna (Italy); UPN: Universidad Pública de Navarra, Navarra (Spain); USV: Unitat de Sanitat Vegetal, Servei de Laboratoris de Sanitat Agrària, DARP, Barcelona (Spain); PMV: Laboratoire de Pathologie Moleculaire et Végétale INRA/INA-PG, Paris (France)

[b] avirulent mutant (*hrp*$^-$,*dsp*$^-$::MudIIPR13, Cm^r) of strain CFBP1430 from Laboratoire de Pathologie Moleculaire et Végétale INRA/INA-PG, Paris (France)

2.3. REDUCTION OF *E. AMYLOVORA* POPULATION ARTIFICIALLY INOCULATED IN PEAR BUDWOODS AND PLANTS BY THERMOTHERAPY

In order to evaluate the survival of *E. amylovora* population after heat treatment, pear branches and plants were bacteria inoculated and heat treated. Bacteria were externally (as epiphyte) and internally (as endophyte) inoculated in budwoods of branches, whereas pear plants were only internally inoculated with bacteria. As Spain is considered a protected zone for fire blight, plant material was artificially inoculated in laboratory under biological controlled conditions.

2.3.1. Plant material

Pear branches (20-25 cm long, 6-8 buds) of cv. Conference and cv. Abate Fetel were collected at A phenological stage from different commercial orchards of Girona (Spain). Pear self-rooted plants of cv. Conference (CAV clone) obtained by micropropagation were also used. Plants containing dormant leaf buds were 2 to 3 years old, about 30 cm high and grown in 1 L plastic containers.

2.3.2. Bacterial strains and growth conditions

Strain PMV6076 of *E. amylovora*, an avirulent mutant (*hrp*$^-$, *dsp*$^-$::MudIIPR13, Cm^r) of strain CFBP1430, and a spontaneous mutant resistant to 50 μg mL^{-1} of rifampicin (Rif^r) of the same strain were used in some experiments. In other experiments, a spontaneous mutant resistant to 100 μg mL^{-1} of rifampicin of strain ESP101 was used. The strains were grown on LB agar supplemented with 50 or 100 μg mL^{-1} of rifampicin, at 25 ºC for 24 h. Suspensions of 10^8 to 10^9 cfu mL^{-1} from cultures grown on LB agar at 25 ºC for 24 h were inoculated on plant material.

2.3.3. Bacterial inoculation

Pear branches were externally and internally inoculated with *E. amylovora,* while pear plants were only internally inoculated.

External inoculation. 10 cm length fragments of branches were soaked for 1 min in the bacterial suspension. After inoculation, cuttings were dried for 30 to 45 min to allow pathogen adhesion to the shoot surface. Three replicates of three fragments per treatment were used and the experiment was repeated twice. The two replicates of the experiment consisted of inoculation on branches of cultivars Conference and Abate Fetel, respectively.

Figure 1.1. Internal inoculation of *E. amylovora* in pear buds.

Internal inoculation. For internal inoculations, *E. amylovora* suspensions were injected in the bud with a 1 mL syringe. The needle of the syringe was carefully introduced into the bud through the distal end until half of its longitudinal axis was reached (Figure 1.1). Five buds were inoculated per branch or plant twig. Five repetitions of one branch or plant were used. Inoculated plant material was introduced into sealed plastic bags for heat treatment. Bacterial population levels were determined before and after heat exposure.

2.3.4. Heat treatment

Pear branches received dry and moist heat at 45 ºC and 50 ºC as described in p. 26. Heat exposure time differed according to the type of bacteria inoculation. Surface inoculated shoots were exposed 0, 45 and 60 min at 45 ºC, and 0, 15 and 30 min at 50 ºC. Internally inoculated shoots were exposed 0, 60 and 90 min at 45 ºC, and 0, 30 and 60 min at 50 ºC. Heating times were higher in internally inoculated buds because it was considered that internal bacteria are more protected than epiphytic populations. Plants were only treated with moist heat. Pear plants were wrapped in moistened absorbent paper; one end of the paper was dipped into water (for assuring moist conditions) and maintained in an incubator (MLR 350H, Sanyo, Japan) at selected temperatures (45 ºC or 50 ºC). Roots of plants were protected with absorbent paper in a polyethylene bag. Heat treatments were carried out by maintaining the plants in an incubator for 2, 4, 6 and 8 h at 45 ºC, and 1, 2 and 3 h at 50 ºC. After heat treatment, the plants were placed in an environmental chamber at 22-25 ºC, 12 h photoperiod and 70-80% relative humidity. Control plants were inoculated with pathogen and not exposed to heat.

2.3.5. Assessment of bacterial population

Bacterial population was assessed before and after heat treatments. Each externally inoculated branch fragment was weighed and transferred into a plastic tub containing 20 mL of sterile distilled water, then placed in a rotary shaker at 150 rpm for 30 min for external washing of bacterium and the supernatant was series diluted tenfold. Bacterial population was determined by plating 20 μL of corresponding dilutions onto LB agar plates supplemented with 10 μg mL^{-1} cloramphenicol or with 10 μg mL^{-1} cloramphenicol and 50 μg mL^{-1} rifampicin, depending on the strain used. Plates were incubated at 25 ºC for 48 h. Results were expressed as cfu per gram of fresh weight. Five

internally inoculated buds were placed into plastic bags containing 10 mL of extracting buffer (saline phosphate buffer pH 7.2, 2% polyvinylpyrrolidone 10, 1% manitol, 10 mM reduced glutathione), and grinded in a lab blender (Stomacher, IUL Instruments, Germany) for 2 min. Aliquots and serial dilutions were subsequently plated on LB, supplemented with 100 $\mu g\ mL^{-1}$ of rifampicin. In pear plants, samples were taken at regular intervals and the bacterial population was determined. Five buds of each plant shoot were transferred into plastic bags containing 4 mL of extracting buffer and grinded in a lab blender for 2 min. Aliquots and serial dilutions were subsequently plated on LB, supplemented with 100 $\mu g\ mL^{-1}$ of rifampicin and incubated 24-48 h at 25 ºC. Results were expressed as cfu per bud.

2.3.6. Assessment of disease incidence after heat treatment

Heat treated shoots with internally inoculated buds were forced to sprout as previously described (p. 27). Shooting conditions were favorable for the disease development. One month after the heat treatment, the percentage of swollen shoot buds and the disease incidence (percentage of infected shoots) were determined.

2.3.7. Experimental design and data analysis

A randomized factorial design with three factors (heat, temperature and exposure time) was carried out. Each treatment consisted of three replicates. The effect of factors on variables was determined by analysis of covariance (ANCOVA) where the exposure time was considered as a covariate, using the GLM procedure of SAS (Version 8.2, SAS Institute Inc., NC, USA). All data sets were tested for equality of variance (Bartlett's test) and normality of errors (Shapiro-Wilk's test). When necessary, appropriate transformations of the values were performed to normalize data and stabilize the variance throughout the data range prior to analysis of variance.

3. RESULTS

3.1. SENSITIVITY OF PLANT MATERIAL TO HEAT TREATMENT

Pear micropropagated plants with dormant buds survived to both, moist and dry heat at 45 ºC and 50 ºC at all exposure times tested (Figure 1.2). ANCOVA indicated a non-significant effect of any parameter (type of heat, temperature and exposure time) on plant shooting in both experiment replicates (P>0.05: R^2= 0.55). In general, the production of tips after heat treatments was better at 45 ºC than at 50 ºC, probably due to the fact that at 50 ºC the upper parts of almost all plants died.

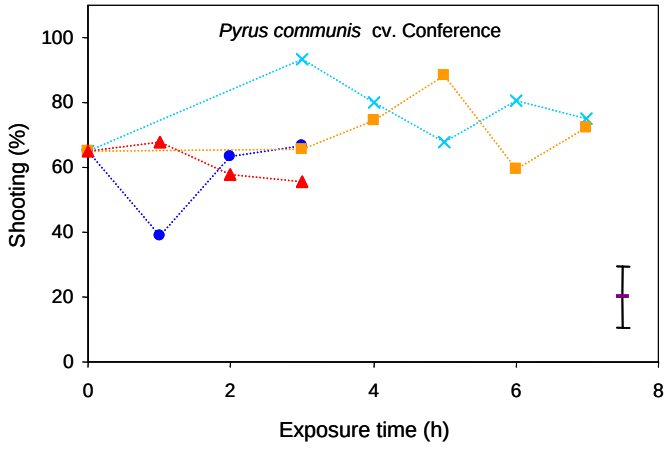

Figure 1.2. Survival of dormant pear plants after heat treatments (▲: dry heat 50 ºC ■: dry heat 45 ºC ●: moist heat 50 ºC x: moist heat 45 ºC). Values are the mean of two experiments and five replicates per experiment. Error bars on the right bottom corner correspond to the mean standard error of all treatments.

Dormant buds of several rosaceous species were tested for their sensitivity to thermotherapy. Survival and shooting of scions after heat exposure depended on the species or cultivar, and on the exposure time (P<0.001: R^2=0.68). Temperature-exposure time interaction also had significant effect on scion survival (P<0.001). Temperature effect (45 ºC or 50 ºC) was related to the exposure time since temperature, *per se*, did not show a significant effect on shooting (P=0.91). Although differences were observed between moist and dry heat treatments, the analysis of covariance indicated a non-significant effect of the type of heat treatment on shooting (P=0.52), so differences were due to the temperature-duration of exposure combination. Most species and cultivars maintained under moist conditions in an incubator survived exposure at 45 ºC for up to 7 h, and at 50 ºC for up to 3 h. Moist heat treatments reduced shooting percentage by up to 20% after 3 h (Figure 1.3). However on scions sealed in polyethylene bags and immersed in a waterbath (dry heat), a satisfactory survival rate was only obtained at 45 ºC for 3 h. Treatments with dry heat at 50

ºC longer than one hour were harmful for most species and cultivars, with a reduction of shooting to 0-30%. No harmful effect of heat was observed in *Cotoneaster horizontalis.*

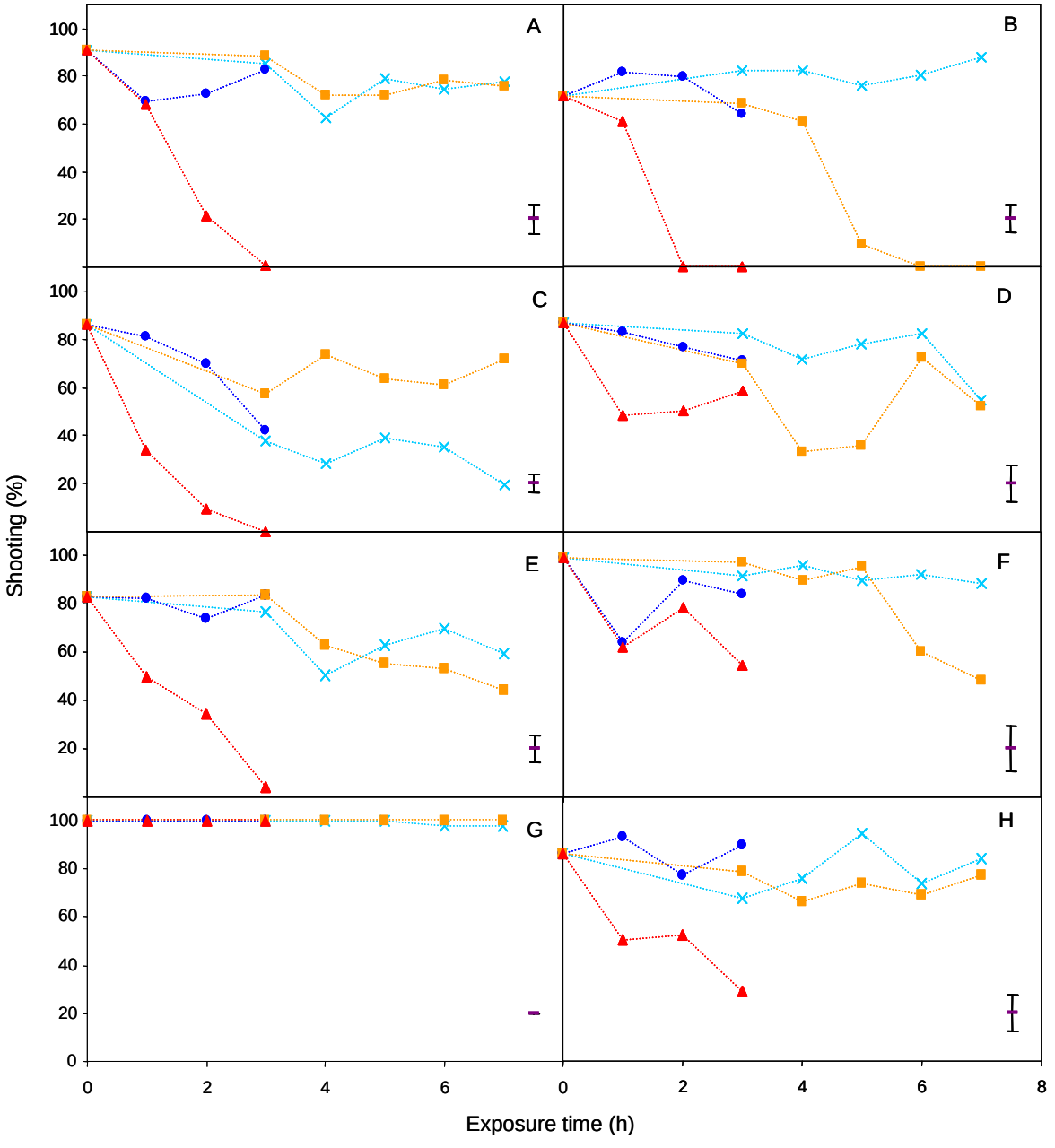

Figure 1.3. Effect of heat on survival of budwoods of different species and cultivars of rosaceous family. **A**: *Pyrus communis* cv. Conference, **B**: *Pyrus communis* cv. Grand Champion, **C**: *Pyrus communis* cv. Abate Fetel, **D**: *Sorbus aria*, **E**: *Cotoneaster dammeri* cv. Skogholm, **F**: *Cotoneaster wardii*, **G**: *Cotoneaster horizontalis,* and **H**: *Crataegus monogyna*. ▲: dry heat 50 ºC ■: dry heat 45 ºC ●: moist heat 50 ºC ×: moist heat 45 ºC. Each value is the mean of five repetitions. Error bars placed on the right bottom corner of each panel correspond to the mean standard error of all treatments for a cultivar or species.

3.2. THERMOSENSITIVITY OF *ERWINIA AMYLOVORA* STRAINS

Covariance analyses indicated a very significant effect of strain, temperature and exposure time on viability of bacterial population (P<0.001: R^2=0.87). Interaction between strain and exposure time also had a significant effect (P=0.01). Triple interaction had no significance (P=0.33). Values of bacterial population recovered after heat treatment are listed in Table 1.4. Strains showed different heat sensitivity depending on temperature and exposure on time. A rapid decrease of bacterial populations occurred at 45 ºC with exposure times higher than 15 min, and at 50 ºC with times higher than 20 min in almost all strains (Figure 1.4). For statistical analysis, no colony-forming units recovered on plates were considered as 4.9 x 10 cfu mL^{-1} resulting in a detection level of 5 x 10 cfu mL^{-1}.

For a more accurate evaluation of heat effect on *E. amylovora* viability, the survival of each strain was calculated as the cfu recovered at each time (t) divided by initial cfu recovered at time 0 min (survival=cfu/cfu_0). The kinetics of bacterial survival followed an exponential model (Figure 1.5). The equation of the exponential model is:

$$y=a \cdot e^{bt}$$

where y is the survival (dependent variable); a represents the initial survival, b is the thermal reduction coefficient rate, and t is the exposure time to heat treatment. To calculate a and b parameters, the exponential model was linearized by natural log (Ln) transformation of dependent variable (survival). The thermal death constants for the exponential reaction model of *E. amylovora* strains are listed in Table 1.5.

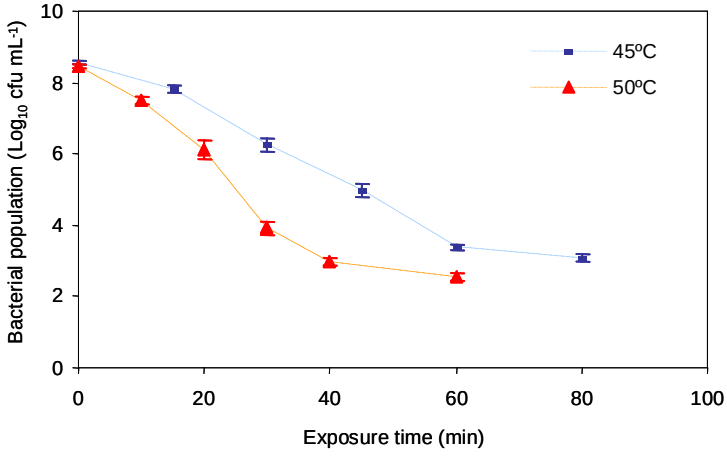

Figure 1.4. Kinetics of reduction of bacterial population levels through exposure times to 45 and 50 ºC. Each value is the mean of 39 strains. Error bars represent the standard error for each treatment.

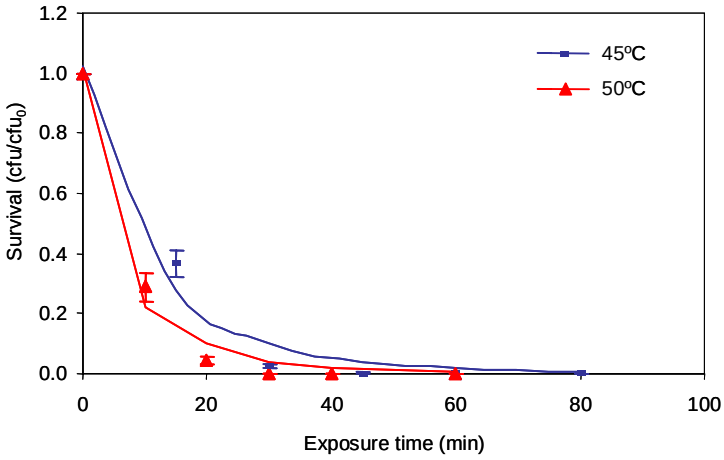

Figure 1.5. Thermal death kinetics of *E. amylovora* at 45 and 50 ºC. Solid lines represent predicted values according the exponential model. Each value is the mean of 39 strains. Error bars represent the standard error.

To evaluate and compare heat sensitivity of 39 strains, lethal thermal time was calculated. The lethal thermal time of a population is the time interval necessary to reduce the number of colony-forming units (cfu) in a 100%, defined in this work as 0.01 of survival (or $LT_{99.9}$) and 0.1 of survival (or LT_{99}). This is a more precise way to evaluate and compare heat susceptibility of various strains. For most of the strains the time required for bacterial population reduction to 99.9% to 50 ºC was 25 min, shorter than to 45 ºC which was 40 min (Table 1.6). Thermal death time ($LT_{99.9}$) of 39 strains did not exceed 55 min at 45 ºC or 37 min at 50 ºC. However, some variations among the strains could be observed. Among the strains the most susceptible at both temperatures were IVIAEa115 and IVIA1614, whereas strains UPN514 and UPN610 were the most resistant at both temperatures. A normal distribution of strains was observed according to their lethal thermal time for 99.9 and 99% death at 45 and 50 ºC (Table 1.7).

Table 1.8 summarizes the exponential model and linearized equation of thermal death kinetics of *E. amylovora* strains and the estimated lethal thermal time for 99.9% and 99% of bacterial population reduction.

Table 1.4. Bacterial population (Log_{10} cfu mL^{-1}) of recovered *E. amylovora* strains after heat treatments

	Temperature (ºC)											
	45						50					
Time (min) / Strain	0	15	30	45	60	80	0	10	20	30	40	60
CFBP1430	8.68[a]	7.95	6.65	4.86	3.34	2.76	8.51	8.12	7.49	3.74	3.47	2.21
CUCM273	9.00	8.25	7.59	6.62	3.68	3.66	8.31	7.82	7.28	3.70	3.03	2.85
EPS101	8.47	7.29	7.18	6.09	2.83	2.20	8.26	8.34	7.62	6.43	3.14	2.73
IVIA1614	8.06	5.35	4.10	3.05	2.08	1.98	8.09	5.89	nd	4.06	1.80	2.59
IVIAEa115	8.65	5.92	3.88	2.70	2.18	<1.69	8.57	7.85	nd	3.10	2.17	<1.69
NCPPB1734	8.16	7.81	6.70	5.70	3.68	3.27	8.49	7.50	6.48	3.07	3.24	3.62
NCPPB1819	8.35	8.18	7.60	6.45	3.70	3.54	8.18	8.20	5.99	4.25	2.15	<1.69
NCPPB2080	8.43	7.82	4.19	3.70	2.96	2.71	8.54	7.00	nd	2.76	3.27	3.11
NCPPB2291	8.22	8.14	7.64	6.70	3.70	3.70	9.14	7.52	5.31	4.33	3.60	3.29
NCPPB2791	8.26	8.28	6.33	6.01	3.65	3.22	8.28	7.83	5.10	3.79	2.47	2.34
NCPPB311	9.10	8.35	7.44	5.20	3.46	3.48	8.79	7.61	6.14	2.55	2.39	1.90
NCPPB3159	9.14	8.31	6.32	4.79	3.34	2.06	8.57	8.22	nd	2.86	2.50	2.56
NCPPB3548	8.67	7.96	6.59	2.92	2.50	1.86	8.75	8.26	nd	3.22	3.02	2.31
NCPPB595	8.39	8.09	6.21	4.17	2.86	1.89	8.45	7.85	6.43	3.17	2.43	2.07
NCPPB683	8.57	8.03	6.20	4.23	3.55	3.20	8.44	7.55	6.22	2.54	3.07	1.88
OMPBO1185	9.07	7.46	3.92	3.60	3.17	2.78	8.56	8.19	nd	4.09	2.92	2.24
PMV6076	8.38	7.77	6.71	4.46	3.02	2.47	8.34	8.24	nd	6.33	3.49	3.22
UPN500	8.39	8.31	7.43	4.49	3.58	3.52	8.46	7.45	nd	4.63	2.40	2.03
UPN506	9.14	8.48	7.46	6.61	3.70	3.70	9.07	8.40	7.54	6.57	3.49	<1.69
UPN513	8.32	8.22	5.45	5.70	3.66	nd	8.61	7.43	5.12	3.49	3.53	3.52
UPN514	8.04	7.52	6.70	5.70	3.70	3.70	7.99	7.50	6.44	3.70	3.22	2.84
UPN524	8.51	8.20	6.70	5.68	3.70	3.68	8.51	7.31	4.28	3.03	3.51	3.14
UPN529	8.62	8.31	6.70	5.70	3.70	3.70	8.48	7.70	6.44	3.38	3.32	3.23
UPN530	9.24	8.32	7.63	6.68	3.70	3.70	8.24	8.28	7.31	6.47	3.67	3.40
UPN536	8.55	8.19	6.67	5.70	3.70	3.65	8.45	6.71	4.80	3.50	3.21	2.51
UPN544	8.47	8.07	5.06	3.56	<1.69	3.70	8.57	7.44	nd	3.19	3.19	2.89
UPN546	8.32	8.09	4.83	3.70	3.39	3.13	8.41	7.12	nd	2.95	3.08	2.85
UPN575	8.35	8.09	6.29	5.24	3.36	2.64	8.25	6.05	4.47	3.70	3.47	3.14
UPN576	8.46	8.24	6.67	5.68	3.68	3.47	8.40	7.68	6.15	3.62	3.22	3.12
UPN588	8.39	8.02	6.70	5.70	3.70	3.70	8.44	7.70	6.44	3.07	2.52	2.55
UPN609	8.34	7.99	6.52	5.28	3.31	2.23	8.81	7.49	6.43	3.36	3.37	2.93
UPN610	8.24	7.90	6.70	5.70	3.70	3.70	7.55	7.26	7.03	3.70	3.70	3.70
UPN611	8.24	8.14	5.98	3.70	3.05	2.48	8.44	7.00	nd	3.15	2.78	2.51
UPNEaZ13	8.54	5.26	4.05	3.87	3.09	3.44	8.52	8.44	nd	6.06	3.47	2.19
UPNEaZ9	9.09	7.47	5.17	4.11	3.68	3.68	8.60	8.40	nd	4.51	3.48	2.90
UPNEaZ7	8.53	7.19	3.88	3.31	3.70	3.34	8.67	8.36	nd	5.16	3.70	2.64
USV1000	8.64	8.48	7.35	5.40	3.72	3.49	8.56	5.39	nd	4.55	2.71	1.80
USV1043	9.25	8.43	7.64	6.41	3.70	3.70	8.54	7.33	nd	6.11	2.85	2.28
USV2194	8.64	8.29	7.21	5.01	3.62	3.70	8.71	5.10	nd	3.70	3.22	2.96
Mean standard error	*0.10*	*0.10*	*0.13*	*0.14*	*0.07*	*0.11*	*0.10*	*0.12*	*0.23*	*0.18*	*0.13*	*0.18*

[a] Values are the mean of three replicates. Detection level was 5 x 10 cfu mL^{-1} and for statistical analysis values lower than detection level were replaced by 4.9 x 10 cfu mL^{-1}(=1.69 log cfu mL^{-1}). nd: not determined

Table 1.5. A and b parameters of exponential model and goodness of fit for thermal response of *E. amylovora* strains at 45 and 50 ºC

	Temperature (ºC)							
	45				50			
Strain	**a**[a]	**b**	**R^2**	***P***	**a**[a]	**b**	**R^2**	***P***
CFBP1430	1.523	-0.187	0.97	0.000	1.420	-0.274	0.90	0.004
CUCM273	2.168	-0.172	0.92	0.003	1.190	-0.249	0.83	0.011
EPS101	2.483	-0.192	0.91	0.003	5.258	-0.251	0.87	0.007
IVIA1614	0.075	-0.170	0.88	0.006	0.124	-0.222	0.82	0.035
IVIAEa115	0.054	-0.196	0.87	0.007	0.509	-0.299	0.89	0.016
NCPPB311	0.852	-0.197	0.94	0.001	0.426	-0.294	0.85	0.009
NCPPB595	2.271	-0.209	0.97	0.000	0.530	-0.279	0.87	0.020
NCPPB683	0.963	-0.174	0.93	0.002	0.493	-0.276	0.86	0.008
NCPPB1734	2.258	-0.158	0.96	0.001	0.231	-0.216	0.74	0.029
NCPPB1819	4.357	-0.161	0.89	0.005	2.423	-0.295	0.91	0.003
NCPPB2080	0.330	-0.181	0.84	0.010	0.125*	-0.224	0.76	0.053
NCPPB2291	4.947	-0.154	0.86	0.008	0.163	-0.234	0.88	0.006
NCPPB2791	2.664	-0.164	0.93	0.002	0.536	-0.260	0.88	0.006
NCPPB3159	1.235	-0.217	0.99	0.000	0.467	-0.274	0.80	0.039
NCPPB3548	1.288	-0.226	0.91	0.003	0.510	-0.284	0.87	0.022
OMPBO1185	0.082	-0.187	0.81	0.015	0.910	-0.276	0.92	0.010
PMV6076	1.870	-0.192	0.96	0.001	3.355	-0.232	0.89	0.005
UPN500	2.561	-0.172	0.88	0.005	0.653	-0.271	0.93	0.007
UPN506	2.055	-0.176	0.92	0.002	3.723	-0.303	0.95	0.001
UPN513	2.072	-0.182	0.89	0.017	0.123	-0.209	0.76	0.023
UPN514	1.649	-0.142	0.94	0.001	0.860	-0.228	0.87	0.007
UPN524	1.733	-0.161	0.94	0.001	0.083	-0.213	0.72	0.033
UPN529	1.668	-0.164	0.94	0.001	0.417	-0.231	0.81	0.014
UPN530	1.774	-0.177	0.92	0.002	3.228	-0.219	0.88	0.005
UPN536	1.654	-0.162	0.94	0.001	0.113	-0.228	0.87	0.007
UPN544	0.428	-0.181	0.74	0.028	0.242	-0.240	0.83	0.032
UPN546	0.563	-0.170	0.84	0.010	0.195	-0.232	0.81	0.038
UPN575	2.219	-0.183	0.97	0.000	0.055	-0.185	0.78	0.020
UPN576	2.056	-0.165	0.95	0.001	0.431	-0.231	0.84	0.010
UPN588	1.689	-0.156	0.94	0.001	0.547	-0.264	0.83	0.012
UPN609	2.867	-0.192	0.98	0.000	0.123	-0.238	0.82	0.013
UPN610	1.828	-0.151	0.94	0.001	0.705	-0.180	0.79	0.019
UPN611	1.658	-0.192	0.92	0.002	0.200	-0.245	0.85	0.025
UPNEaZ7	0.098	-0.152	0.73	0.030	1.393	-0.257	0.96	0.004
UPNEaZ9	0.127	-0.164	0.84	0.010	1.045	-0.251	0.91	0.012
UPNEaZ13	0.021	-0.133	0.69	0.040	2.933	-0.271	0.95	0.005
USV1000	2.724	-0.175	0.94	0.002	0.089	-0.240	0.89	0.016
USV1043	1.540	-0.183	0.94	0.002	0.939	-0.254	0.92	0.011
USV2194	1.767	-0.170	0.92	0.003	0.026*	-0.197	0.74	0.061

[a] estimated *a* and *b* parameters of exponential equation $y=a\,e^{bt}$ by linear regression of linearized equation, R^2: determination coefficient, *P*: significance level. *Strain with low fit

Table 1.6. *In vitro* lethal thermal times (LT, min) to achieve a 99.9% or 99% of bacterial mortality for *E. amylovora* strains at 45 and 50 ºC

	45 ºC			50 ºC	
Strain	**$LT_{99.9}$ [a]**	**LT_{99}**	**strain**	**$LT_{99.9}$ [a]**	**LT_{99}**
NCPPB2291	55.3	40.3	UPN530	36.9	26.4
UPN514	52.1	35.9	UPN610	36.3	23.6
NCPPB1819	52.0	37.7	PMV6076	35.0	25.1
UPN610	49.8	34.6	EPS101	34.1	25.0
NCPPB1734	48.9	34.3	UPN514	29.6	19.5
NCPPB2791	48.2	34.1	UPNEaZ13	29.5	21.0
UPN588	47.8	33.0	CUCM273	28.4	19.2
UPN524	46.5	32.1	UPNEaZ7	28.1	19.2
UPN576	46.3	32.3	UPNEaZ9	27.6	18.5
UPN536	45.8	31.6	UPN506	27.1	19.5
UPN500	45.6	32.2	USV1043	26.9	17.9
UPN529	45.3	31.3	CFBP1430	26.5	18.1
USV1000	45.2	32.1	NCPPB1819	26.4	18.6
CUCM273	44.6	31.2	UPN576	26.3	16.3
USV2194	44.0	30.5	UPN529	26.1	16.1
UPN506	43.2	30.2	NCPPB1734	25.2	14.5
UPN530	42.3	29.3	OMPBO1185	24.7	16.3
UPN575	42.2	29.6	NCPPB2791	24.2	15.3
UPN513	42.1	29.4	UPN500	23.9	15.4
UPN609	41.6	29.5	UPN588	23.9	15.2
EPS101	40.7	28.7	UPN513	23.1	12.0
USV1043	40.2	27.6	UPN544	22.9	13.3
NCPPB683	39.4	26.2	UPN546	22.7	12.8
PMV6076	39.3	27.3	NCPPB683	22.5	14.1
CFBP1430	39.2	26.9	NCPPB595	22.4	14.2
UPN611	38.6	26.6	NCPPB3159	22.4	14.0
UPN546	37.3	23.8	NCPPB3548	22.0	13.9
NCPPB595	36.9	25.9	NCPPB2291	21.8	12.0
NCPPB311	34.3	22.6	IVIA1614	21.7	11.3
UPN544	33.5	20.7	UPN611	21.6	12.2
NCPPB3159	32.7	22.2	UPN575	21.6	9.2
NCPPB2080	32.1	19.4	NCPPB2080	21.6	11.3
NCPPB3548	31.7	21.5	IVIAEa115	20.8	13.1
UPNEaZ7	30.2	15.0	UPN524	20.8	10.0
UPNEaZ9	29.5	15.5	UPN536	20.7	10.6
IVIA1614	25.4	11.8	NCPPB311	20.6	12.8
OMPBO1185	23.6	11.3	UPN609	20.2	10.5
UPNEaZ13	23.0	5.7	USV1000	18.7	9.1
IVIAEa115	20.4	8.6	USV2194	16.5	4.8
Mean	*39.9*	*26.6*	*Mean*	*24.9*	*15.4*

[a] $LT_{99.9}$ lethal time for survival of 0.1%; LT_{99}: lethal time for survival of 1%

Table 1.7. Frequency distribution (number of strains) of *E. amylovora* according to their lethal thermal time for 99.9 and 99% death at 45 and 50 ºC

	Temperature (ºC)			
	45		50	
Letal thermal time (min)	$LT_{99.9}$	LT_{99}	$LT_{99.9}$	LT_{99}
55-59	1			
50-54	3			
45-49	10			
40-44	8	1		
35-39	6	3	3	
30-34	7	14	2	
25-29	1	9	12	3
20-24	3	5	20	4
15-19		3	2	13
10-14		2		16
5-9		2		3

Table 1.8. Exponential model, linear equations, and *in vitro* lethal thermal times (LT, min) for *E. amylovora* at two temperatures (45 and 50 ºC)

Temperature (ºC)	Exponential model[a]	Linearized equation	$LT_{99.9}$	LT_{99}	R^2	*P*
45	$y=1.03*e^{(-0.17*t)}$	Ln y=0.03-0.17*t	40.8	27.3	0.83	<0.001
50	$y=0.48*e^{(-0.25*t)}$	Ln y=-0.73-0.25*t	24.7	15.5	0.81	<0.001

[a] Model and values are calculated from pooled data of 39 strains; y: survival as cfu/cfu_0. Exponential model parameters were obtained by linear regression of linearized equation with survival values of 39 strains and three replicates.

3.3. REDUCTION OF *E. AMYLOVORA* POPULATION ARTIFICIALLY INOCULATED ON PEAR BUDWOODS AND PLANTS BY THERMOTHERAPY

Plant material was externally inoculated by immersion of cutting fragments in an *E. amylovora* suspension and internally inoculated by injection of *E. amylovora* into the buds. Effect of heat treatment on viability of surface and internal *E. amylovora* populations was determined.

As it was observed that the cultivar had a significant effect ($P<0.001$: $R^2=0.89$), data from surface inoculated pear cuttings of cv. Conference and cv. Abate Fetel were analyzed separately. A very significant effect of exposure time ($P<0.001$: $R^2=0.77$), of temperature-time interaction ($P=0.003$) and heat-time interaction ($P=0.005$) on reduction of bacterial population after heat treatment was obtained in cv. Conference. In cv. Abate Fetel, exposure time and temperature-time interaction had a very significant effect ($P<0.001$: $R^2=0.92$) on reduction of bacterial population. The kinetics of

bacterial population reduction were similar for the two cultivars, although in cv. Abate Fetel the initial bacterial population was one log higher than in the cv. Conference, probably due to the different morphology of the cuttings (Figure 1.6). 60 min of treatment at 45 ºC in both heat types (moist and dry) reduced bacterial population to the limit detection level, whereas 30 min at 50 ºC reduced *E. amylovora* population by two or three logs. Dry heat reduced bacterial population to lower levels than moist heat. In non-treated controls, the population levels remained constant at their initial level throughout the time (data not shown). To compare the effectiveness of treatments, the time required to reduce bacterial population levels to 1 cfu g^{-1} f.w. was estimated by linear regression ($P<0.001$: $R^2>0.65$) (Table 1.9). Longer times were required for moist heat at 45 ºC (273-137 min), and these were significantly different from dry heat at 50 ºC (62-55 min).

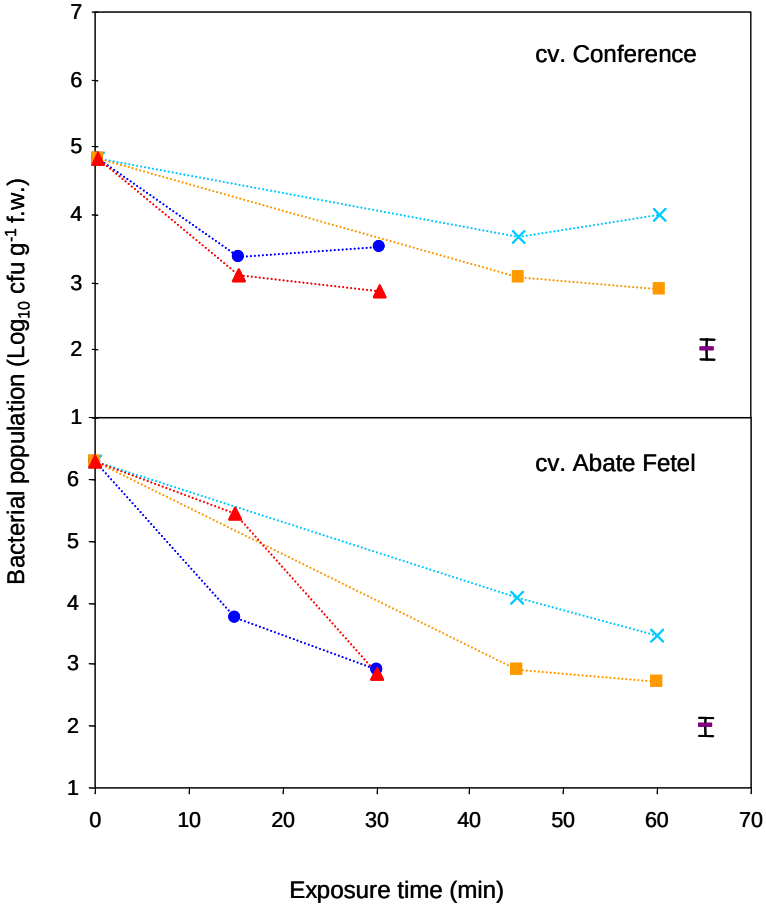

Figure 1.6. Effect of heat treatment on reduction of *E. amylovora* population surface-inoculated in buds of pear cuttings. ▲: dry heat 50 ºC ■: dry heat 45 ºC ●: moist heat 50 ºC x: moist heat 45 ºC. Values are the mean of three replicates. Error bars placed on the right bottom corner correspond to the mean standard error of all treatments.

Table 1.9. Exposure time (min) for reduction of surface-inoculated *E. amylovora* population to 1 cfu g^{-1} f.w. on pear cuttings at 45 and 50 ºC

	Pear cultivar	
Heat conditions	**Conference**	**Abate Fetel**
Moist heat 45 ºC	273.4[a] a	137.1 a
Moist heat 50 ºC	150.8 b	51.6 c
Dry heat 45 ºC	149.4 b	105.6 b
Dry heat 50 ºC	62.1 c	55.4 c

[a] estimated exposure time calculated by linear regression. Values with the same letter did not differ significantly according to Tukey's test (P=0.05). Values are the mean of three replicates.

Reduction of internal *E. amylovora* population on pear budwoods after heat treatment was significantly affected by the exposure time of heat treatment (P<0.001: R^2=0.90). No significant effect of either type of heat or temperature was observed (P>0.05). Maximal exposure times tested (60 min for 50 ºC and 90 min for 45 ºC) were not enough to totally eradicate the internal population of *E. amylovora* (Figure 1.7 A). Maximal reduction in population levels was obtained after 30-60 min at 50 ºC and 60-90 min at 45 ºC. In both cases populations were reduced by 3 logs.

Shooting of pear branches (cv. Conference) after heat treatment and the effect of heat treatment on shoot infection by *E. amylovora* were also determined. Variance analyses indicated that the type of heat and the exposure time had a significant effect on shooting (P<0.01: R^2=0.84). Interaction between these two factors and between temperature and time also had a significant effect on shooting percentage. Artificially inoculated and heat treated budwoods survived exposure at 50 ºC moist heat up to 60 min, while more than 30 min exposure to the other heat and temperature conditions reduced shooting to 50% from that of non-heat-treated scions (Figure 1.7 B). Variance analyses indicated a very significant effect of exposure time on disease incidence (P<0.001: R^2=0.61). Heat exposures longer than 60 min (except dry and moist heat at 45 ºC which needed 90 min) reduced *E. amylovora* infection from 60% (non-heat-treated plants) to 25-0% (Figure 1.7 C).

In order to evaluate the effect of thermotherapy in the reduction of pathogen population internally inoculated on micropropagated pear plants, two trials were carried out. Trial effect was tested and no significance was observed (P=0.09: R^2=0.66), so data were joined. In these experiments only moist heat were tested. Variance analyses with pooled data from two trials, did not show any significant effect of temperature and exposure time on reduction of bacterial population (P>0.05: R^2=0.60). After 8 h of exposure to 45 ºC in a moist atmosphere in the incubator, no viable bacteria could be detected, whereas incubations for 1-3 h at 50 ºC did not result in the total destruction of the pathogen (Figure 1.8 A). In the second trial, survival of pear plants and disease incidence were also evaluated. Covariance analyses indicated that no factor (temperature and exposure time) had a significant effect on shooting of plant material (P>0.05: R^2=0.55). Shooting values of non-heat treated plants were slightly lower (10%) from those of heat treated plants treated (40%) (Figure 1.8 B). However, covariance analyses indicated a very significant effect of exposure time on disease incidence (P<0.001: R^2=0.59). An increased exposure time reduced disease incidence from 60%

(non-treated control) to 20% in pear plants after 4-8 h at 45 ºC, and 2-4 h at 50 ºC (Figure 1.8 C). Disease symptoms were observed as necrosis or bacterial ooze on non-heat treated pear buds after incubation (Figure 1.9).

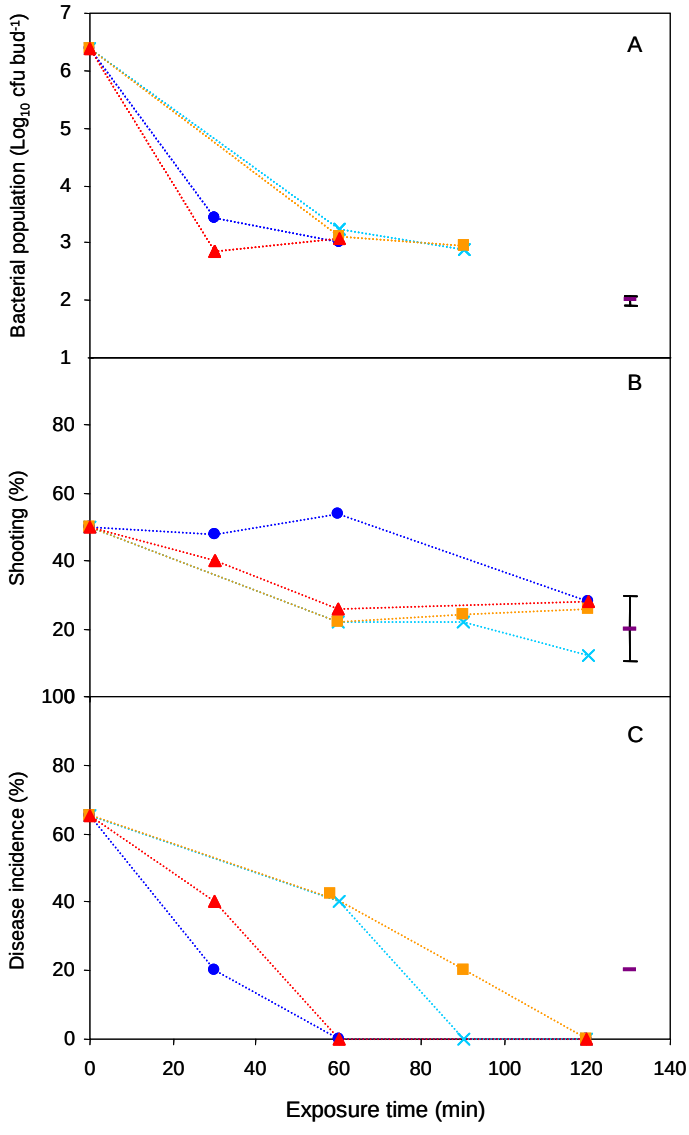

Figure 1.7. Effect of heat treatment on reduction of *E. amylovora* population (**A**), on shooting (**B**), and on disease incidence (**C**) of pear budwoods cv. Conference internally inoculated with the pathogen (▲: dry heat 50 ºC ■: dry heat 45 ºC ●: moist heat 50 ºC x: moist heat 45 ºC). Values are the mean of five replicates. Error bars placed on the right bottom corner correspond to the mean standard error of all treatments.

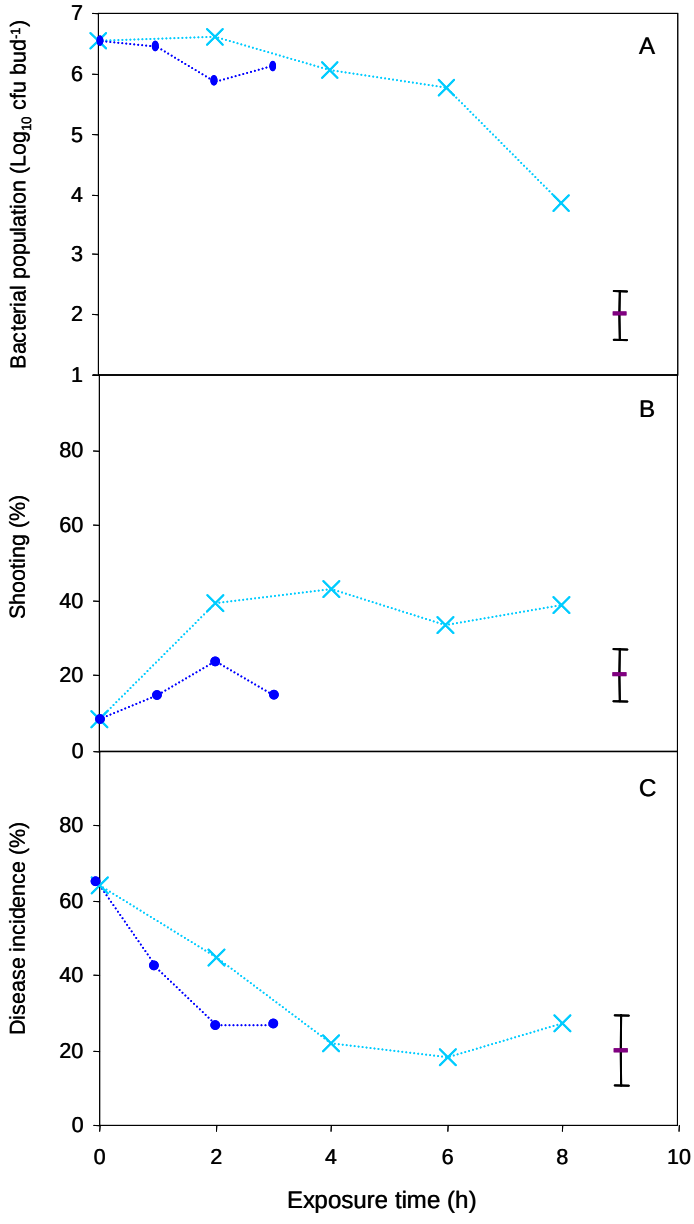

Figure 1.8. Effect of heat treatment on reduction of *E. amylovora* population (**A**), on shooting (**B**) and on disease incidence (**C**) of pear plants cv. Conference internally inoculated with the pathogen (•: moist heat 50 ºC x: moist heat 45 ºC). Values are the mean of two trials with eight repetitions per treatment. Error bars placed on the right bottom corner correspond to the mean standard error of all treatments.

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Figure 1.9. Ooze production and disease symptoms in *E. amylovora*-inoculated and non-heat-treated pear plants.

4. DISCUSSION

Very little data have been published which compare different methods of eliminating viruses or bacteria from woody plants. Our study has demonstrated that heat is a viable way of eradicating *E. amylovora* from the propagation material of the pear.

Although some treatments reduced viability of plant material, successful results were obtained either by increased temperatures or exposure time in branches and plants. The heat resistance of budwoods seemed to vary according to the type of cultivar or species. These results are in agreement with those of Campbell (1970) who found that the ability of woody plants to withstand high temperatures varies greatly and different cultivars show different sensitivity to heat.

Combinations of time-temperature not injurious to the host but effective enough to reduce the internal bacterial population were determined. Our results indicated that almost all plant material tested maintained under moist conditions survived 7 hours at 45 ºC and up to 3 hours at 50 ºC, while more than 1 hour of exposure at 50 ºC with dry heat injured plants and reduced shooting. Similar results were obtained by Aldwinckle and Gustafson (1993) who observed that when thermotherapy was applied to scion budsticks by immersion in hot water, 50% of buds of apple cvs. Idared and Empire survived 60 min at 48 ºC, but all buds were killed by 30 min at 50 ºC.

From previous works it was known that direct immersion of budwoods in hot water or incubation in dry heat was harmful to plant material (Keck *et al*., 1995). Therefore, the thermotherapy methods employed in our study were chosen to provide a gentler form of heat. Nevertheless, when comparing the two methods, some differences in the damaging effect of plant tissues were noted: the moist heat was less harmful than dry heat. This difference might be due to the way in which loss of water from plant tissues was tentatively limited. In the waterbath, sealed polyethylene bags prevented the contact of water and plant tissues and some tissue desiccation could occur as Keck *et al.* (1995) suggested. In plants, the procedure used to protect the roots from heat seems effective, since no damaging effect in shooting was found.

Strains of *Erwinia amylovora* varied in their *in vitro* sensitivity to heat. Although the variability of thermosensitivity seems to be related to the origin of strain, this study showed that *E. amylovora* strains from different geographical and host origins were similar in their thermosensitivity. The limit of bacterial detection of the counting method used in our experiments corresponds to 50 cfu mL^{-1}. Therefore, we cannot conclude that the treatments totally eliminated all the pathogen population, but they rather reduced them to non-detectable level. The time required to reduce the survival of strains at minimal detection level was estimated in 40 min at 45 ºC and 25 min at 50 ºC, while for most strains the time required to reduce them to non-detectable level was up to 80 min at 45 ºC and up to 60 min at 50 ºC. This correlated well with results obtained by Keck *et al*. (1995) who found that 45 ºC for 70 min or 50 ºC for 50 min were enough to destroy pure culture of the bacteria with some variations among strains. These authors tested a small number of strains (8 strains). *In*

vitro survival of *E. amylovora* to thermotherapy may not accurately reflect the survival of a pathogenic bacterium in plant tissues, where the source of nutrients and the host, are in a continually dynamic state because of its growth. Several authors have demonstrated that the heat susceptibility of bacteria depends on the age of the culture used to determine the thermal death point (Grondeau, 1994). Several day-old cultures are more resistant to heat and have a thermal death point superior to 24 to 48 hours than older cultures.

Time required to reduce bacterial population *in vivo* on internally inoculated plant material (branches and plants) was higher than the lethal thermal time determined *in vitro* or *in vivo* on surface inoculated plant material. This is probably due to the fact that in order to eradicate endophytous bacteria heat must penetrate the external layers of plant organs (buds, twigs), and this is only achieved after a certain time of exposure, so latent bacteria in plant material may be more resistant to heat. The preliminary *in vivo* experiments in apple shoots (Eden-Green, 1972) suggested that relative growth rates of *Erwinia amylovora* at different temperatures may not be greatly different from those in broth cultures (Billing, 1974). In our work 30 min at 50 ºC or 60 min at 45 ºC reduced bacterial population to non-detectable levels (5 x 10^2 cfu g^{-1} f.w.) on external inoculated branches. In internally inoculated plants the time required for bacterial population reduction was 8 hours at 45 ºC or up to 3 hours at 50 ºC.

This study showed that *Erwinia amylovora* strains from different origins were similar in their thermosensitivity and that it was possible to reduce sharply and to eliminate in certain circumstances the pathogenic population inside plant tissues. Therefore in the case of *E. amylovora,* the use of heat may be a possible procedure for the treatment of propagating plant material as it has been proposed for other bacteria (Burr *et al*., 1989). Further investigations should help to achieve more precision in the determination of optimal temperatures and the lengths of treatments. The possibility of such a treatment in practical nursery conditions remains to be thoroughly examined. The use of the thermotherapy would be especially recommended prior to the release of fire blight resistant pear, apple and other rosaceous scions from breeding stations where they were selected as resistant plants, and which, if grown in infected areas, or even in contaminated orchards, and could transmit pathogenic populations without showing any symptoms.

However, further tests are needed to establish the total eradication of the pathogen and to determine the effect of thermotherapy on crop emergence and its use under commercial conditions.

CHAPTER 2

IMPROVEMENT OF CHEMICAL CONTROL OF FIRE BLIGHT

1. INTRODUCTION

Chemicals are applied either to eliminate or to inactivate plant-pathogenic bacteria before these bacteria succeed in penetrating the host tissues. They are also applied to render the plant surfaces unsuitable for the establishment of new infections (Psallidas and Tsiantos, 2000). In chemical control of fire blight, a large number of compounds have been tested. Van der Zwet and Keil (1979) summarized the data published (from 1920 to 1979) on chemical control of fire blight on apple and pear, and grouped these products into four categories: copper compounds, antibiotics, carbamates and miscellaneous compounds. The two groups of chemicals that play the most important role in controlling fire blight are copper derivatives and antibiotics which are recommended according to the specific legal restrictions of each country. Two antibiotics are now available for fire blight control (Garret, 1990): streptomycin that is registered is some countries (USA, Canada, New Zealand, and The Netherlands) and flumequine in France and Belgium. Copper compounds are widely used; however the risk of phytotoxicity must be assessed. Other compounds, which have shown a moderate efficacy in experimental assays (e.g. fosetyl-Al), are likely to be available soon for *E. amylovora* control (Table 2.1). However, their use is limited by factors such as a moderate efficacy, or phytotoxicity to the plant.

Recently, the introduction of commercial plant defense activators provides a new option for chemical management of plant diseases based on the induction of host resistance. New compounds could be used against fire blight, overcoming some of the disadvantages of copper and antibiotics. This group of product includes fosetyl-Al (a phosphonate derivative), oxolinic acid (a quinolone family compound), prohexadione calcium (a plant growth regulator), acibenzolar-S-methyl (a benzothiadiazole derivate) and harpin (an *E. amylovora* protein). It needs to be noted that, of the vast number of chemicals tested against fire blight, only a few have been registered for control of fire blight in countries with fire blight history (Vanneste, 2000).

Table 2.1. Efficacy of chemical compounds used for fire blight control in field experiments (Kooistra and Gruyter, 1984; Paulin *et al.* 1990; Lindow *et al*., 1996)

Product	Efficacy (%)
Copper derivatives	36-78
Antibiotics	70-94
Streptomycin	77-94
Kasugamycin	80-82
Flumequine	70-74
Fosetyl-Al	40-50

1.1. Conventional compounds

1.1.1. Copper derivatives

Copper compounds were the first biocides used for disease control and are the only bactericides registered for their use in most crops. Although these materials are chemically stable and are not readily washed from plants, control often has been incomplete when they are applied as protectants or as eradicants of bacterial diseases (Andersen *et al.,* 1991). Copper derivatives have been extensively used to reduce losses due to *Erwinia* spp. in many crops and they have also been used to reduce fire blight of fruit trees (Loper *et al*., 1991; Saygili and Üstün, 1996). Cooper compounds have been established as effective bactericides and have been used against fire blight on apple and pear since 1900 (van der Zwet and Keil, 1979). The active ingredient of these products is the copper ion. Copper is both an essential micronutrient and a toxic heavy metal for most living cells. Copper ranks after silver (Ag) and mercury (Hg) for cell toxicity. Because of its high phytotoxicity, copper was not used as a foliar pesticide until 1885, when the French scientist Millardet accidentally observed that a mixture of copper sulfate ($CuSO_4$) and lime ($Ca(OH)_2$) was not phytotoxic but exhibited a fungicidal action against *Plasmopara viticola* on grapevine. The effectiveness of copper against various pathogens is attributed to the availability of copper ions that inactivate many different enzymes and other proteins essential to cell membrane function. This broad mode of action is not only restricted to microorganisms but also can damage foliage and fruits in the host plant. The copper formulations used for control of bacterial diseases in crops include copper sulfates, copper oxychlorides, and copper oxides. Copper products registered for use as fungicides or bactericides are low in phytotoxicity, have small particle sizes to optimize their availability, and have materials added to improve spreading and sticking. Most copper fungicides and bactericides contain at least 20-50% copper. They act by releasing copper ions at levels that are toxic to fungi and bacteria, but do not affect the plant cells. Copper compounds are quite good bactericides, but their use may have phytotoxic effects on leaves and fruits; therefore they are employed primarily before bloom, by combined strategies with other compounds or in recuced spraying programs (Ninot *et al.*, 2002)

1.1.2. Antibiotics

Antibiotics are organic compounds produced by microorganisms which selectively inhibit the growth of other microorganisms by inhibition of nucleic acid metabolism enzyme activity. The use of antibiotics against bacterial pathogens of cultivated plants started in 1950 (Psadillas and Tsiantos, 2000). Fire blight was among the first bacterial diseases for which antibiotics were tested, since the use of copper compounds was restricted because of phytotoxicity. Indeed, the first experiments on fire blight control with streptomycin were performed in USA in the early 1950s and, by the end of that decade, streptomycin was widely used in apple and pear orchards. Other antibiotics such as oxytretacycline, kasugamycin and gentamicin sulfate are also effective against *E. amylovora* (Aldwinckle and Norelli, 1990; Saygili and Üstün, 1996; Spitko and Alvarado, 1999). It has been reported that *E. amylovora* is sensitive to many antibiotics *in vitro* (Paulin, 2000) but only a few of them have practical value for field applications (streptomycin, oxytretacycline and kasugamycin). Streptomycin gives good control, but is not registered for use in Spain and in many other countries. In Spain, kasugamycin is the only one antibiotic allowed for crop protection.

1.1.3. Limitations of copper derivatives and antibiotics as used in control of bacterial diseases

The effectiveness of copper derivatives for control of certain plant bacterial diseases is reduced by the selection of copper-resistant bacterial strains and phytotoxic effects on plant. Copper-resistant strains of *Xanthomonas campestris* pv. *vesicatoria*, *Pseudomonas syringae* pv. *tomato* (Cooksey, 1990) and *Pseudomonas syringae* pv. *syringae* (Sundin *et al.*, 1989) have been isolated in USA. However, copper resistant strains of *Erwinia amylovora* have not yet been described. The mechanisms of copper resistance in phytopathogenic bacteria are not known, and little information on bacterial copper resistance mechanisms is available (Cha and Cooksey, 1991). Therefore, copper is recommended for fire blight control, but when it is used in blossom period, it causes phytotoxity. Although more expensive than copper compounds, streptomycin normally does not cause russetting. The use of antibiotics, however, has two major drawbacks. Antibiotics, especially streptomycin, are used in human and animal medicine, so their use for plant protection is under strict regulation and in some countries is not allowed (e.g. in France, Spain, Italy and Switzerland). Secondly, repeated and frequent use of antibiotics often leads to the selection of antibiotic resistant strains of *E. amylovora*. Such strains were detected in the early 1970s in some areas of the USA, and later they were found in New Zealand, Greece, Israel and other countries (Moller *et al*., 1981; McManus and Jones, 1994; Manulis *et al*., 1998). The main limitations of antibiotic use are: the selection for resistance in pathogen and its transfer among diverse bacterial populations, plant or animals; their toxicity; the short persistence on the plant surfaces under field conditions; and the lack of complete systemic activity.

1.2. Alternative compounds

1.2.1. Phosphonate derivatives

Phosphonates are a class of chemical compounds derived from phosphorous acid based on O-P (fosetyl-Al) or C-P bonds (ethephon), some of which have applications in crop protection. Phosphonates are characterized by the presence of one or more $–C-PO_3-H_2$ groups. Fosetyl-Al is a systemic fungicide active against oomycetes pythiaceous fungi (*Phytophtora* and *Pythium* spp.) (Bompeix *et al.*, 1980). It is used to control a wide range of fungal diseases, mainly of horticultural crops. This fungicide is unique because is ambimobile, it has both acropetal and basipetal mobility. It is able to move both up and downwards in the plant making it particularly suitable for the control of root and foliar diseases. Fosetyl-Al does not show high antifungal and antibacterial activity *in vitro* and its mode of action has been extensively investigated. Initially it was believed that it stimulated plant defense mechanisms (Bompeix *et al*., 1981; Guest, 1984), but subsequent work indicated that it was converted within the plant to the fungitoxic phosphonic acid (phosphorous acid, H_3PO_3) (Fenn and Coffey, 1985). It is now generally accepted that phosphonic acid is responsible for the activity of fosetyl-Al against phytopathogenic fungi, although the involvement of plant defense systems is difficult to exclude (Guest and Grant, 1991). So, the mode of action of fosetyl-Al against fungi is not clear. Research by Fenn and Coffey (1984) supported a direct mode of action, but Guest (1986) reported that host defense response to infection by *Phytophthora* spp. increased when plants were treated with fosetyl-Al and postulated that a combination of direct and indirect activity accounted for the control of *Phytophtora* diseases. Overall it appears that a combination of direct and indirect actions is needed to explain the efficacy of fosetyl-Al against the oomycetes. The activity of fosetyl-Al against phytopathogenic bacteria also appears to be both direct and indirect. Fosetyl-Al was moderately effective in the control of bacterial diseases as citrus canker (McGuire, 1988), fire blight of pear (Paulin *et al*., 1990), and some bacterial diseases of ornamental plants (Chase, 1993). *In vitro* tests indicated a variable effect of fosetyl-Al with a significant inhibition of growth of *Xanthomonas* spp. (most sensitive) and *Pseudomonas cichorii* (moderately sensitive), and slight growth inhibition of *Erwinia* spp. (least sensitive) (Chase, 1993) and *Pseudomonas syringae* pv. s*yringae* (Moragrega *et al*., 1998). The main advantage of this complex mode of action is that phosphonate resistant pathogens do not appear to have arisen after 23 years of use (Guest *et al.*, 1995). An effect that must be considered is the phytotoxicity when fosetyl-Al was applied to plants that were also treated with copper compounds, either separately or together (Chase, 1989). Fosetyl-Al is commercially available as systemic fungicide under the trade names of Aliette® or Alerte® (Aventis CropScience, SA).

Ethephon is an organic phosphorus compound, ethylene generator and plant growth regulator. Its use varies with plant species, chemical concentration, and time of application. Ethephon regulates plant growth and development by application to various growth sites. Additionally, it is an active ingredient of a variety of commercial herbicides. Ethephon's mode of action is via liberation of

ethylene, which is absorbed by the plant and interferes in the growth process (Kidd and James, 1991). In the last 10 years it has been demonstrated the role of ethylene as a signal involved in disease resistance. Lawton *et al.* (1994) reported that ethephon stimulated two independent signal transduction pathways for plant resistance by different mechanisms. One is the induction of systemic acquired resistance (SAR) gene expression dependent on the accumulation of salicylic acid (SA) and the other is an ethylene signal transduction distinct pathway. In plant, ethephon spontaneously degrades and releases ethylene gas (C_2H_4), hydrochloric acid (HCl) and phosphonic acid (H_3PO_3) (Yang, 1969). In soil, a rapid degradation to phosphoric acid, ethylene, and chloride ions was reported (Hartley and Kidd, 1987). Additionally it has been reported that ethephon gives some degree of protection on plants against viral infection (Bellés *et al.*, 1990). Ethephon is commercially available as plant growth regulator under different trade names among them Ethrel® (Aventis CropScience, SA)

1.2.2. Benzothiadiazole

The syntethic compound benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) or acibenzolar-S-methyl (ASM) is the first product belonging to a new generation of crop protectants effective through plant resistance activation (Friedrich *et al.,* 1996; Görlach *et al.,* 1996). BTH is an inducer of systemic acquired resistance (SAR) in several annual plants. The chemical has no known direct antifungal effects and is thought to mimic salicylic acid in the signal transduction pathway that leads to systemic acquired resistance. BTH is a true SAR compound by the same signal transduction pathway as pathogen inoculation. To be considered as a SAR inducer, a chemical should fulfill at least the following three criteria (Sticher *et al.*, 1997): (a) show no direct antimicrobial activity, (b) protect against a range of pathogens without specificity, (c) activate host defense mechanisms which are similar to those induced systematically after biological activation of SAR and even in tissues not confronted by the SAR inducer. These mechanisms include cell wall reinforcement and the systemic accumulation of pathogenesis-related (PR) proteins. Within this concept, the benzothiadiazole compound acibenzolar-S-methyl has been developed and commercialized for the control of cereal powdery mildew in Europe (Ruess *et al*., 1996). BTH was obtained through screening compounds structurally related to salicylic acid and is able to induce resistance in a wide range of plants (Tally *et al.*, 1999). Sauerborn *et al.* (2002) showed that the phenomenon of induced resistance is not restricted to viral, bacterial and fungal diseases and demonstrated the great potential of this protection strategy as an effective component of future plant production systems. Extensive experimental analysis of SAR in model plant species such as tobacco and *Arabidopsis* (Delaney *et al.*, 1995; Sticher *et al.*, 1997) have been carried out, but relatively little information is available on induced resistance in perennial woody crops.

Induced resistance (IR) by means of synthetic inducers, in some crop systems, is a suitable strategy to enhance the plant's natural defense responses to control pathogens. In several systems BTH induced a set of 'SAR genes' including members of PR families. The same genes were also induced by pathogen attack and by biological inducers of SAR (Ward *et al*., 1991; Uknes

et al., 1992; Friedrich *et al*., 1996; Lawton *et al*., 1996). Induced resistance by BTH has been demonstrated in over 30 crops, including annual and perennial, monocotyledonous and dicotyledonous plants against a broad spectrum of leaf and root pathogens.

BTH has shown moderate efficacy (50%) in control of some bacterial and fungal diseases in annual and perennial plants. Although in powdery mildew of cucumber BTH has been ineffective by prolonged use and additionally phytotoxic (Table 2.2). It indicates that in each disease, the product must be assayed and optimized.

Table 2.2. Efficacy of benzothiadiazole in different pathosystems

Pathosystem and disease	Efficacy (%)	Reference
Bacterial Diseases		
Erwinia amylovora-apple Fire blight	50-75	Brisset *et al.,* 2000
Xanthomonas campestris pv. *vesicatoria*-pepper Pepper bacterial spot	60	Buonaurio *et al.,* 2002
Fungal Diseases		
Colletotrichum gloeosporioides-cashew Anthracnose	50	Lopez and Lucas, 2002
Gymnosporangium asiaticum-Japanese pear Japanese pear rust	50	Ishii *et al.,* 1999
Venturia nashicola-Japanese pear Japanese pear scab	50	Ishii *et al.,* 1999
Peronospora parasitica-cauliflower Downy mildew	50	Ziadi *et al.,* 2001
Sphaerotheca fuliginea-cucumber Powdery mildew	Ineffective[a]	Wurms *et al.,* 1999
Magnaporthe grisea-rice Rice blast	19-66	Schweizer *et al.,* 1999

[a] In addition, prolonged use of BTH resulted in phytotoxic symptoms

BTH is commercially released in some countries as a plant health promoter or plant activator of annual crops and is marketed in some countries under the trade name of Bion® (marketed in Europe by Novartis Crop Protection) or Actigard® (marketed in the USA by Syngenta). Since 1996 it has been commercialized in some countries (Germany and Switzerland) for the control of various disease crops (powdery mildew in wheat). In Spain is not registered for its use in agriculture and is only allowed for experimental use.

1.2.3. Use of plant defense inducer chemicals in control of fire blight

Over the past twenty years, induced disease resistance was demonstrated in a number of plant-pathogen systems by using biotic and abiotic inducing agents (Sticher *et al.*, 1997). The major hallmark of this type of resistance is the ability of plants to defend themselves against a broad spectrum of pathogens by triggering plant species specific defense responses (Métraux, 2001).

These new products, which give promising results and could be used in fire blight control, can be mixed or combined with copper derivatives or streptomycin, to overcome their limitations. Some of these new compounds, which have been registered in some countries, are fosetyl-Al (Aliette®) and oxolinic acid (Starner®). Other compounds not yet registered for control of bacterial diseases include benzothiadiazole (Bion®, Actigard®), prohexadione-Ca (Apogee®, Regalis®) and harpin (Messenger®). Induced resistance may provide an alternative approach to plant protection especially when control is not achieved by conventional methods.

The novel mode of action of plant defense activators suggests that they will be of great potential value in the integrated control of plant diseases, especially where resistance to conventional fungicides is a problem. As these compounds act on the endogenous defense pathways of the plant, they provide broad spectrum disease control which should be durable. There are many unresolved questions about their use, however, such as optimal timing and method of application on different crops, integration with other types of pesticides, and interactions with the physiology of the plant. Many factors still limit the use of such compounds, in particular their partial effectiveness. Moreover, many work remains to be developed to improve the formulation and also to determine the amounts and the best moments of application. Like other bacterial diseases, fire blight is difficult to control but the induction of resistance may be a new strategy for disease control.
Some studies have been performed with BTH, but still remains to be tested on pear or other host plants such as ornamentals. However, further data will be needed on the stability of protection, dose-response and an appropriate spray schedule before the practical application of BTH in the orchard for fire blight control.

OBJECTIVES

The aim of this study was to evaluate the efficacy of several chemicals including copper derivatives and plant defense inducers (phosphonates and benzothiadiazole) in the fire blight control in apple and pear. For each product the optimal dose and timing were determined in order to obtain the maximal reduction of disease levels. In addition, an *in vitro* test was performed to investigate the direct effect of products on bacterial growth.

2.1. EFFICACY OF COPPER DERIVATIVES IN FIRE BLIGHT CONTROL

Several experiments were carried out to determine the effect of copper derivatives, copper-mancozeb mixtures and antibiotics in the infection of *Erwinia amylovora*. Five copper derivatives were compared at different doses and timing of applications. Additionally a mixture of copper and mancozeb and two antibiotics were tested. Efficacy of chemicals was evaluated on pear and apple under controlled environment conditions.

2.1.1. Plant material

Two types of plant material were used: pear and apple potted plants and detached branches forced to bloom. Self-rooted plants of pear cv. Conference (CAV clone) obtained by micropropagation (Agromillora Catalana, SA, Barcelona, Spain) and apple cv. Fuji on M-9 EMLA rootstocks (BC2 infel 7690 clone from Certiplant, SL, Lleida, Spain) were used. Plants were 2 to 3 years old, about 30 cm high, and were grown in 1 L plastic pots filled with a commercial peatmoss/vermiculite/perlite potting mix (type BVU, Prodeasa, Girona, Spain). Plants were chilled during winter, pruned and forced to bud in the greenhouse, 2 to 3 months before experiments began. Actively growing plants were maintained in the greenhouse and fertilized once a week with a solution of 200 ppm N-P-K (20-10-20).

Chemical treatments with copper compounds (but not with copper-mancozeb mixture) were also performed on flowers developed on detached branches of pear cv. Conference and apple cv. Fuji. Branches were collected in winter at A phenological stage and forced to bloom as described by Montesinos and Vilardell (1991).

2.1.2. Products

Products used were copper derivatives: Bordeaux mixture (20% metallic copper), Bordeaux mixture (25%), copper oxide (50%), copper hydroxide (50%) and copper oxychloride (50%); two copper-mancozeb mixtures (matured and not-matured) and two antibiotics (streptomycin sulfate and kasugamycin). The chemicals tested and their sources are listed in Table 2.3. The products were selected because they are commonly used and more or less effective in fire blight control. The copper-mancozeb mixture was introduced in the experiments because it is used for control of other bacterial diseases (bacterial spot of tomato and pepper and bacterial speck of tomato). Antibiotics were used as reference products; the streptomycin is widely used in fire blight control but not allowed in Spain, and Kasugamycin is allowed for control of some plant diseases, and can be used in Spain.

Table 2.3. Source and molecular formula of chemicals evaluated for fire blight control under controlled environment conditions

Active ingredient	Trade name	Source[a]	Molecular formula
Copper derivatives			
Bordeaux mixture	Caldo bordelés Vallés® (Wettable powder, 20%)	IQV, SA	$CuSO_4.3Cu(OH)_2.3CaSO_4$
Bordeaux mixture	Caldo bordelés 25 Vallés® (Wettable powder, 25%)	IQV, SA	$CuSO_4.3Cu(OH)_2.3CaSO_4$
Copper hydroxide	Hidrocobre 50 Vallés® (Wettable powder, 50%)	IQV, SA	$Cu(OH)_2$
Copper oxide	Cobre Rojo Vallés® (Wettable powder, 50%)	IQV, SA	CuO
Copper oxychloride	Curenox 50® (Wettable powder, 50%)	IQV, SA	$CuCl_2Cu(OH)_2$
Dithiocarbamate			
Mancozeb	Dithane DG® (Wettable powder, 80%)	Dow Agrosciences, SA	$(C_4H_6MnN_2S_4)_x$ $(Zn)_y$
Antibiotics			
Streptomycin sulfate	Streptomycin® (Salt, 100%)	Sigma, SA	$C_{21}H_{39}N_7O_{12}$
Kasugamycin	Kasugamycin (Wettable powder, 50%)	Lainco, SA	$C_{14}H_{25}N_3O_9$

[a] IQV, SA: Industrias Químicas del Vallés, SA, Barcelona, Spain

2.1.3. Product application

Plants and flowers on detached branches were uniformly sprayed to runoff with aqueous solution of products (or water for the control) using a pressurized sprayer at 400 kPa. Two doses of copper were evaluated: 1.5 and 3 g Cu L^{-1} in all products. The copper-mancozeb mixture consisted of 2.5 g Cu L^{-1} plus 1.5 g mancozeb L^{-1} prepared just before its application or the same copper-mancozeb mixture prepared 10 hours before spraying plants (matured mixture). Each experiment included water-sprayed controls and antibiotics (streptomycin 100 mg a.i. L^{-1} and kasugamycin 30 mg a.i. L^{-1}) as effective reference products in controlling the disease. For each product three strategies of application were tested: preventive, protective and curative. The preventive strategy consisted of product application before wounding plant organs and bacterial inoculation. In protective strategy the products were applied after wounding plant organs but before pathogen inoculation, while in curative strategy the pathogen was wound-inoculated in plants before the products were sprayed. In all experiments products were applied 2 hours before or after plant wounding or pathogen inoculation, according to strategy (Table 2.4).

Whole plant experiments. Two trials were carried out, which included the five copper derivatives, the copper-mancozeb mixture and the two antibiotics. Copper derivatives were applied at 1.5 and 3 g Cu L^{-1} and following the three previously described strategies: preventive, protective and

curative. The copper-mancozeb mixture was applied in preventive and protective strategies (Table 2.4).

Detached branch assays. Five copper derivates (1.5 and 3 g Cu L^{-1}) and two antibiotics were applied following strategies preventive and curative (Table 2.4).

Table 2.4. Products, doses and strategies of application tested for fire blight control under controlled environment on pear and apple plants and branches

Product	Dose	Strategy of application[a]		
Whole plant assays				
Copper compounds		preventive	protective	curative
Copper derivatives	1.5 and 3g Cu L^{-1}	+	+	+
Mixture				
Copper - mancozeb (matured)[b]	2.5 g Cu L^{-1} - 1.5 g a.i. L^{-1}	+	+	-
Copper - mancozeb	2.5 g Cu L^{-1} - 1.5 g a.i. L^{-1}	+	+	-
Antibiotics				
Streptomycin sulfate	100 mg L^{-1}	+	+	+
Kasugamycin	30 mg L^{-1}	+	+	+
Detached branch assays				
Copper compounds				
Copper derivatives	1.5 and 3g Cu L^{-1}	+	-	+
Antibiotics				
Streptomycin sulfate	100 mg L^{-1}	+	-	+
Kasugamycin	30 mg L^{-1}	+	-	+

[a] preventive: product application before wounding plant organs and bacterial inoculation, protective: products were applied after wounding plant organs but before pathogen inoculation, and curative: pathogen was wounding inoculated in plants before product spray. In detached branches no wound was performed on flowers before bacteria inoculation. + tested, – not tested
[b] prepared 10 hours before sprays

2.1.4. Bacterial inoculum

The virulent strain EPS101 of *E. amylovora* isolated from infected tissues in a commercial pear orchard located in Lleida (Catalunya, Spain) was used. Bacterial suspensions were prepared in sterile distilled water from Luria-Bertani (LB) (Maniatis *et al*., 1982) agar cultures grown at 25 ºC for 24 h. Suspensions at 10^8 cfu mL^{-1} were prepared just before the inoculation and maintained at 4 ºC until their use. Concentration was adjusted from absorbances at 620 nm and interpolation in a previously obtained calibration curve. Dilution plating was used to determine the final concentration of the inoculum suspensions.

2.1.5. Inoculation

Leaves on pear plants were inoculated with *E. amylovora* by means of a brush dipped into the bacterial suspension. Prior to inoculation, a cut was made in the leaf with scissors to allow the pathogen to enter plant tissues. The three youngest leaves in a shoot were inoculated. Two or, when available, three shoots were inoculated per plant, and three replicates of three plants were used for each treatment. Flowers on detached branches were also inoculated with a brush dipped into bacterial suspension and introduced into the receptacle (calyx cup) of the flower. No lesion was produced to flowers before bacteria inoculation. One flower of each cluster and five clusters per branch were inoculated. Each treatment was composed of three repetitions of three branches. Inoculated plant material was sealed into internally wet plastic bags and incubated at 25 ºC, 70-80% relative humidity and 16 hours light photoperiod, in a controlled environment chamber (PGR15, Conviron, Canada). All inoculum management and inoculations were made inside a laminar flow biological safety cabinet (NU-425, NuAire Inc., MN, USA), to avoid the spread of a quarantine pathogen.

2.1.6. Disease assessment

In plants, disease intensity was rated using a visual 0 to 4 scale of increasing necrosis progression as follows: 0=no symptoms; 1=necrosis located at the inoculation point and the midvein; 2=necrosis affecting the midvein and the petiole; 3=necrosis expanding through the shoot; 4=necrosis affecting the shoot and other leaves down in the shoot (Duron *et al*., 1987). Symptoms were scored 10 days (pear) or 15 days (apple) after inoculation.

In flowers, disease intensity was also scored using a 0 to 4 arbitrary scale based on the progression of the necrosis throughout the flower as shown in Figure 2.1. 0=no symptoms; 1=necrosis visible only in the calyx cup; 2=necrosis progressing through the receptacle; 3=necrosis progressing down the stem to the fruit spur or twig; 4=necrosis affecting other flowers of the cluster. Symptoms were scored at 7 days (pear) or 12 days (apple) after inoculation.

The disease severity (S) was calculated for each plant or branch according to the following formula:

$$S = \sum_{n=1}^{N} \frac{I_n}{(N \cdot I_{max})} \cdot 100$$

where, I_n is the corresponding rating of infection in an inoculated leaf/flower, N is the number of leaves or flowers inoculated per plant or branch, I_{max} is the maximum disease rate (4).

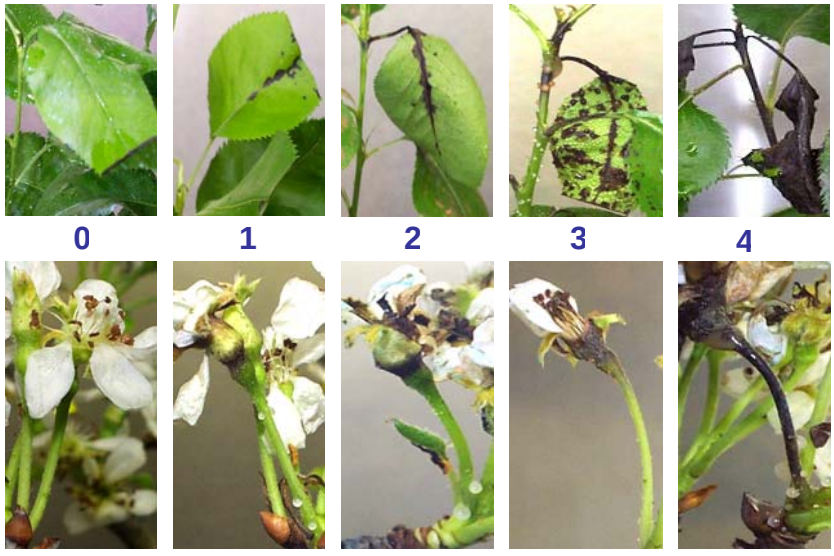


Figure 2.1. Infection levels and disease indexes used to assess the disease intensity in pear and apple leaves and flowers.

2.1.7. Experimental design

The experimental design consisted of a randomized factorial design composed of three factors (product, dose and strategy). Three replicates of three plants or branches were included in each treatment.

2.1.8. Data analysis

The effect of product, dose and spraying strategy on disease severity was determined by analysis of variance (ANOVA) using the general linear models (GLM) procedure of statistical analysis system (SAS) (Version 8.2, SAS Institute Inc., NC, USA). All data sets were tested for equality of variance (Bartlett's test) and normality (Shapiro-Wilk's test). Differences among treatments were identified using the Tukey's test. When necessary, appropriate transformations (square root of severity) were performed to normalize data and stabilize the variance throughout the data range prior to analysis of variance.

2.2. EFFICACY OF PLANT DEFENSE ACTIVATORS IN FIRE BLIGHT CONTROL

Two types of systemic compounds were evaluated: phosphonate derivatives and benzothiadiazole. These compounds were compared with copper and antibiotics in their efficacy. Product timing and optimal dose were determined for each product.

2.2.1. Plant material

Pear plants cv. Conference (CAV clone) obtained by micropropagation (Agromillora Catalana, SA, Barcelona, Spain) were used. Plants were 2 to 3 years old, about 30 cm high and were grown and maintained as described for previous experiments. One month before starting the experiment, plants were fertilized with an N-K solution (20-20), without phosphorous to prevent interference of phosphate in the phosphonate derivative trials.

2.2.2. Products

Phosphonate derivatives used were: aluminium tris-o-ethylphosphonate (fosetyl-Al, Alerte®, Rhône-Poulenc Agro, France) and 2-chloroethylphosphonate (ethephon, Ethrel 48®, Compagnie Française de Produits Industriels, France). The benzothiadiazole derivative used was benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester (acibenzolar-S-methyl, Bion®, Syngenta Agro, SA, Spain) (Table 2.5). The products were selected because they seem to have a common mode of action, acting as inducers of systemic acquired resistance in plants. The two phosphonate derivatives have a common phosphonate group, but they differ in their chemical structure. The concentration of phosphonate derivatives is expressed as $[HPO_3]^{2-}$ content. Values given can be converted to the corresponding weight of active ingredient by multiplication using the following conversion factors: 1.38 (for fosetyl-Al) and 1.81 (for ethephon). The benzothiadiazole is structural and functionally homologous to salicylic acid. Streptomycin, kasugamycin and copper oxychloride were used as reference products.

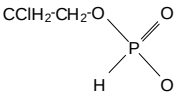
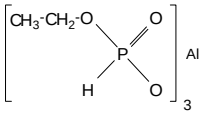
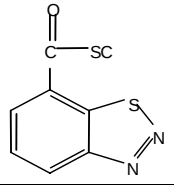
2.2.3. Product application

Chemicals were applied by foliar spraying to runoff in aqueous solutions at the desired dose, with a 1 L hand sprayer. Sprays were done at different timings, depending on the assay, before pathogen inoculation. After spraying with the chemicals, the plants were allowed to dry until no liquid droplets were visible. Non-treated-control plants were sprayed with water.

In a preliminary experiment, chemicals were compared for their efficacy in fire blight control, and they were sprayed at time interval and dose recommended by manufacturers for other bacterial diseases. The recommended dose of fosetyl-Al for plant bacterial disease control is 1.9-3.8 g a.i. L^{-1} (1.4-2.8 g HPO_3^{2-} L^{-1}) (Chase, 1993), so fosetyl-Al 1.86 g HPO_3^{2-} L^{-1} (2.57 g a.i. L^{-1}) and ethephon 0.5 g HPO_3^{2-} L^{-1} (0.9 g a.i. L^{-1}) were applied following two different timings: (1) 7 and 2 days before pathogen inoculation, and (2) each day during the 5 previous days to bacteria inoculation. Copper

(1.5 g Cu L^{-1}) and streptomycin (100 mg a.i. L^{-1}), were sprayed 2 hours before pathogen inoculation. The experiment was repeated three times (Table 2.6).

Table 2.5. Products, source and structural formula of plant defense activators tested for fire blight control

Active ingredient	Common and trade name	Source	Structural formula
Phosphonate derivatives			
Aluminium tris-o-ethylphosphonate	Fosetyl-Al, Alerte® (Wettable powder, 80%)	Rhône-Poulenc Agro	
2-chloroethylphosphonate	Ethephon, Ethrel 48® (Liquid, 48%)	Compagnie Française de Produits Industriels	
Benzothiadiazole			
Benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester (BTH)	Acibenzolar-S-methyl (ASM), Bion® (Granule, 50%)	Syngenta Agro, SA	
Antibiotics			
Streptomycin sulfate	Streptomycin (Powder, 100%)	Sigma, SA	
Kasugamycin	Kasugamycin (Wettable powder, 50%)	Lainco, SA	
Copper			
Copper oxychloride	Cobreluq-50® (Wettable powder, 50%)	Lérida Unión Química, SA	

Two experiments were performed to determine the optimal spraying schedule and dose for fosetyl-Al and ethephon, and two additional experiments for benzothiadiazole. The optimal spraying time before pathogen inoculation was determined. Seven spray times were tested for fosetyl-Al (1.86 g HPO_3^{2-} L^{-1}) and ethephon (0.5 g HPO_3^{2-} L^{-1}) which were applied once 7, 6, 5, 4, 3, 2 or 1 day before pathogen inoculation. To determine the optimal dose of phosphonates in fire blight control, only fosetyl-Al was tested at 0.62, 0.93, 1.86, 3.72 g HPO_3^{2-} L^{-1} applied a week before bacteria inoculation. The optimal time of application was determined for benzothiadiazole (100 mg a.i. L^{-1}) by spraying 10, 8, 7, 6, 5, 4, 3, 2 or 1 day before pathogen inoculation. A second experiment was conducted to evaluate the effect of BTH concentration on disease levels. Six doses of benzothiadiazole were tested (250, 200, 175, 150, 125, 100 and 75 mg a.i. L^{-1}) which were sprayed once 7 days before *E. amylovora* inoculation.

Table 2.6. Product, dose and schedule of timing in experiments performed to compare the efficacy of plant defense activators, copper and antibiotics in fire blight control

Product	Dose	Number and timing of sprays (time before bacteria inoculation)
Experiments 1 and 2		
Fosetyl-Al	1.86 g HPO_3^{2-} L^{-1}	two (7 and 2 days)
Fosetyl-Al	1.86 g HPO_3^{2-} L^{-1}	five (5, 4, 3, 2 and 1 day)
Ethephon	0.5 g HPO_3^{2-} L^{-1}	two (7 and 2 days)
Ethephon	0.5 g HPO_3^{2-} L^{-1}	five (5, 4, 3, 2 and 1 day)
Benzothiadiazole	100 mg a.i. L^{-1}	two (7 and 2 days)
Streptomycin	100 mg a.i. L^{-1}	one (2 h)
Copper oxychloride	1.5 g Cu L^{-1}	one (2 h)
Experiment 3		
Fosetyl-Al	1.86 g HPO_3^{2-} L^{-1}	two (7 and 2)
Ethephon	0.5 g HPO_3^{2-} L^{-1}	two (7 and 2)
Benzothiadiazole	100 mg a.i. L^{-1}	two (7 and 2)
Kasugamycin	30 mg a.i. L^{-1}	one (2 h)
Streptomycin	100 mg a.i. L^{-1}	one (2 h)
Copper oxychloride	1.5 g Cu L^{-1}	one (2 h)

2.2.4. Bacteria inoculation

Vigorously growing shoots at least 30 cm of height from 2 to 3 year-old plants were inoculated by cutting the 3 youngest leaves in a shoot with a pair of scissors dipped into a 10^8 cfu mL^{-1} suspension of *E. amylovora* EPS101 (Norelli *et al.*, 1984). Inoculated plants were sealed in plastic bags and incubated at the optimal conditions for the development of the disease as previously described (p. 57).

2.2.5. Disease assessment

Disease was evaluated 7 days after incubation. Disease severity was assessed by using a visual 0 to 4 scale of increasing necrosis progression throughout leaves from inoculation point, as described in page 57.

2.2.6. Experimental design and data analysis

The experimental design consisted of a completely randomized factorial design with three replicates of three plants per treatment. The effect of treatment on disease severity was determined by analysis of variance (ANOVA) using the general linear models (GLM) procedure of statistical analysis system (SAS) (Version 8.2, SAS Institute Inc., NC, USA). All data sets were tested for equality of variance (Bartlett's test) and normality (Shapiro-Wilk's test). When necessary, appropriate transformations of the values were performed to normalize data and stabilize the variance throughout the data range prior to analysis of variance. Means were separated by the Tukey's test. The optimal dose of product for disease control, was determined by linear regression and the REG procedure of SAS was used.

2.3. Efficacy of plant defense activators combined with copper and antibiotics in fire blight control

The purpose of these experiments was to evaluate the efficacy of simultaneous application (mixed strategy) and consecutive application (combined strategy) of products with different activity (copper sulfate, antibiotics, fosetyl-Al and benzothiadiazole) in fire blight control.

Several greenhouse experiments and field trials were carried out in order to determine if sprays of plant defense inducers, such as fosetyl-Al and benzothiadiazole, combined or mixed with antibiotics or copper derivatives increased their efficacy in control of *E. amylovora* and permitted a reduction in antibiotic or copper doses. Additionally, an experimental biological control agent was combined with chemicals and was tested. For this purpose, either a mixture in which all active ingredients were added to the same water solution and simultaneously sprayed a week before bacteria inoculation, or different water solutions were prepared for each product and they were consecutively sprayed, plant defense inducers a week before bacteria inoculation and copper and antibiotics two hours before inoculation. Field trials and greenhouse experiments were performed in France, at the INRA Center in Angers, where field inoculations of *E. amylovora* are allowed. Controlled environment chamber experiments were done in the Universitat de Girona.

2.3.1. Plant material

Self-rooted plants of pear cv. Conference (CAV clone) obtained by micropropagation were used in controlled environment experiments. Plants from 2 to 3 years old, and were maintained and grown as previously described (p. 54). Pear and apple seedlings (2-10 leaves) from open-pollinated cv. Kirchensaller and cv. Golden Delicious, respectively, were used in experiments performed at the Angers greenhouse. Plants were grown in individual pots in the greenhouse at 17-22 ºC, under natural photoperiod during spring. The orchard trial was conducted on adult apple trees cv. Gala grafted on M9 rootstock.

2.3.2. Products

Five products were used in both mixed or combined strategy experiments; copper sulfate (Bordeaux mixture, 20% metallic copper); two antibiotics, streptomycin (Streptomycin sulfate, 100%) and kasugamycin (kasugamycin, 50%); two plant defense inducers, fosetyl-Al (Alerte, 80%), and benzothiadiazole (Bion, 50%); and one experimental biocontrol agent. The biological agent was a strain of *Pseudomonas fluorescens* which was isolated at the Universitat de Girona (Spain) and proved highly effective in fire blight control on fruitlets under controlled environment conditions (Cabrefiga, 2000).

Mixtures or combinations of products included a plant defense inducer (fosetyl-Al or benzothiadiazole) and a bactericide (copper sulfate or antibiotics) or the biological control agent. Plant defense inducers were applied at standard dose 1.86 g HPO_3^{2-} L^{-1} for fosetyl-Al and 100-150 mg a.i. L^{-1} for benzothiadiazole. Copper sulfate in mixtures or combinations was sprayed at a

reduced dose (0.37-0.75 g Cu L^{-1} depending on mixtures) and the dose of antibiotics was also reduced from standard (15 mg a.i. L^{-1} for kasugamycin and 40-50 mg a.i. L^{-1} for streptomycin). In all experiments, control plants were sprayed with each product alone or with water (non-treated controls). Different products and doses were combined or mixed depending on the experiment (Tables 2.7 and 2.8). In controlled environment experiments performed in Girona (Spain) products were applied as a mixture or combined, while in the Angers (France) greenhouse and field experiments, products were only combined and the biological agent was included.

Controlled environment chamber experiments. Pear plants were sprayed with a 1 L hand sprayer to runoff with products. Fosetyl-Al and benzothiadiazole were sprayed alone, mixed or combined with antibiotics and copper sulfate reducing by half the concentration of antibiotics and copper sulfate. Products and doses tested are summarized in Table 2.7. Doses of products when applied alone were: fosetyl-Al 1.86 g HPO_3^{2-} L^{-1}, benzothiadiazole 100 mg a.i. L^{-1}, sprayed 7 days prior to inoculation, and kasugamycin 30 mg a.i. L^{-1}, streptomycin 100 mg a.i. L^{-1}, and copper sulfate 0.75 g Cu L^{-1}, sprayed 2 hours before inoculation. In mixed sprays (simultaneously sprayed) a mixture was made with fosetyl-Al (1.86 g HPO_3^{2-} L^{-1}) and kasugamycin (15 mg a.i. L^{-1}), fosetyl-Al (1.86 g HPO_3^{2-} L^{-1}) plus streptomycin (50 mg a.i. L^{-1}), and fosetyl-Al (1.86 g HPO_3^{2-} L^{-1}) plus copper sulfate (0.37 g Cu L^{-1}) and sprayed 7 days before inoculation. In combined product sprays (consecutively sprayed), fosetyl-Al was applied 7 days before inoculation and antibiotics and copper sulfate were applied later, 2 hours before bacteria inoculation. Benzothiadiazole was applied mixed and combined as described for fosetyl-Al. Non-treated control plants were sprayed with water alone. Each experiment consisted of three repetitions of three plants

Greenhouse experiments. Plant defense inducers benzothiadiazole (150 mg a.i. L^{-1}) and fosetyl-Al (1.86 g $HPO_3^{2-}L^{-1}$) were sprayed (alone or combined) two times, 7 and 4 days before bacteria inoculation, while biocontrol agent (7 x 10^8 cfu mL^{-1}), copper sulfate (1.5 g Cu L^{-1}) and kasugamycin (40 mg a.i. L^{-1}) were sprayed 2 hours before pathogen inoculation. Two combined (consecutively sprayed) treatments were carried out: (1) benzothiadiazole and biocontrol agent, and (2) benzothiadiazole and kasugamycin at the same rate and schedule as in treatments alone. Combined products are listed in Table 2.8. Compounds were applied with a 1 L hand sprayer to runoff on apple and pear seedlings. For each treatment, 3 repetitions of 10 plants were used.

Field trial. In the orchard, apple trees were sprayed with products applied to runoff at doses and time intervals previously described for seedlings. Treatments were performed in April at 1-10% bloom. For each treatment, at least 55 clusters of three randomized trees were counted after inoculation.

Table 2.7. Products and doses simultaneously or consecutively sprayed, tested for fire blight control in pear scions under controlled environment experiments performed in Girona (Spain)

Product[a]	**Dose**
Fosetyl-Al	1.86 g HPO_3^{2-} L^{-1}
Benzothiadiazole	100 mg a.i. L^{-1}
Kasugamycin	30 mg a.i. L^{-1}
Streptomycin	100 mg a.i. L^{-1}
Copper sulfate	0.75 g Cu L^{-1}
Fosetyl-Al + kasugamycin	1.86 g HPO_3^{2-} L^{-1} + 15 mg a.i. L^{-1}
Fosetyl-Al + streptomycin	1.86 g HPO_3^{2-} L^{-1} + 50 mg a.i. L^{-1}
Fosetyl-Al + copper sulfate	1.86 g HPO_3^{2-} L^{-1} + 0.37 g Cu L^{-1}
Benzothiadiazole + kasugamycin	100 mg a.i. L^{-1} + 15 mg a.i. L^{-1}
Benzothiadiazole + streptomycin	100 mg a.i. L^{-1} + 50 mg a.i. L^{-1}
Benzothiadiazole + copper sulfate	100 mg a.i. L^{-1} + 0.37g Cu L^{-1}

[a] Products were applied simultaneously as a mixture or consecutively when combined. The mixture was sprayed 7 days before bacteria inoculation. When combined, fosetyl-Al or benzothiadiazole were sprayed 7 days before *E. amylovora* inoculation and copper or antibiotics 2 h before inoculation.

Table 2.8. Products and doses applied consecutively in combined sprays tested for fire blight control in greenhouse and field experiments in Angers (France)

Product[a]	**Dose**
Fosetyl-Al	1.86 g HPO_3^{2-} L^{-1}
Benzothiadiazole	150 mg a.i. L^{-1}
Kasugamycin	40 mg a.i. L^{-1}
Copper sulfate	1.5 g Cu L^{-1}
Biocontrol agent	$7x10^8$ cfu mL^{-1}
Benzothiadiazole + kasugamycin	150 mg a.i. L^{-1} + 40 mg a.i. L^{-1}
Benzothiadiazole + biocontrol agent	150 mg a.i. L^{-1} + $7x10^8$ cfu mL^{-1}

[a] Plant defense inducers (fosetyl-Al and benzothiadiazole) were applied 7 and 4 days before bacteria inoculation. Copper, antibiotics and the biological agent were sprayed 2 h before bacteria inoculation.

2.3.3. Bacteria inoculation

In Girona (Spain) assays, the EPS101 strain of *E. amylovora* adjusted to 10^8 cfu mL^{-1} was used for the inoculations, while in Angers (France) experiments the CFBP1430 strain of *E. amylovora* adjusted to 10^8 cfu mL^{-1} was used. The bacteria were grown at 27 ºC on King's B agar (King *et al*., 1954) for 24 h. Bacterial suspensions were prepared in sterile distilled water to yield a concentration of 10^8 cfu mL^{-1} and used within 30 min.

On pear scions, the 3 youngest leaves in a plant were inoculated by cutting them with a pair scissors dipped into the pathogen suspension. Apple and pear seedlings were inoculated by cutting (1 cm long across the midrib) the youngest nearly expanded leaf in a plant and spraying the

bacterial suspension to runoff on the entire plant 4 hours after wounding. Apple trees were inoculated at full bloom by spraying bacterial suspension to runoff.

2.3.4. Disease assessment

In pear scions, disease symptoms were evaluated 7 days after incubation. In seedling plants, development of fire blight symptoms (petiole and shoot necrosis) was assessed within the 1-2 weeks following *E. amylovora* inoculation. In both experiments, disease severity was quantified by means of a visual 0 to 4 scale of necrosis progression as described previously (p. 57). In orchard trees, the percentage of infected clusters was determined by counting the total number of flower clusters the day after *E. amylovora* inoculation, and the infected clusters 21 days after inoculation.

2.3.5. Experimental design and statistical analysis

The experimental design consisted of a completely randomized factorial design with three replicates per treatment. Most of the experiments were conducted twice. Treatment effects on fire blight severity were determined by analysis of variance (ANOVA) with the SAS system. Means were compared by Tukey's test. Before each analysis, homogeneity of variances and normality of residuals were determined by Bartlett and Shapiro-Wilk tests, respectively. Percentages of disease severity were square root transformed if necessary for statistical analyses.

2.4. *IN VITRO* ANTIBACTERIAL ACTIVITY OF CHEMICALS

The effect of benzothiadiazole, fosetyl-Al, ethephon, copper sulfate, streptomycin and kasugamycin on the growth of 14 strains of plant pathogenic and non-plant-pathogenic bacteria was determined by a growth inhibition agar incorporation test.

2.4.1. Bacterial strains and growth conditions

A total of 14 strains of *Erwinia amylovora*, *E. herbicola*, *Pseudomonas fluorescens*, *P. savastanoi*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *syringae*, *P. syringae* pv. *tomato*, *Serratia marcescens*, *Xanthomonas arboricola* pv. *juglandis*, and *X. vesicatoria* were used (Table 2.9). Strains were isolated from different plant species (pear, peach, olive, bean, tomato, grape, walnut and pepper) and obtained from different source. Bacteria from -80 ºC stored cultures were streaked onto Luria-Bertani agar or yeast extract dextrose calcium carbonate (YDC) agar (Schaad, 1988) depending on the species, and grown at 25 ºC for 48 h. For growth inhibition assays, the low complexing mineral salts medium CYE (casitone yeast extract glycerol) was used (Andersen *et al.,* 1991). Bacterial suspensions (1-5 x 10^9 cfu mL^{-1}) were inoculated onto the agar surface with a sterile toothpick and the plates were incubated at 25 ºC for 5 days.

Table 2.9. List and origin of bacterial species and strains used in test to determine the *in vitro* activity of chemicals

Bacteria	Host plant	Source[a]
Erwinia amylovora EPS101	*Pyrus communis*	INTEA-EPS
E. herbicola EPS21	*Pyrus communis*	INTEA-EPS
E. herbicola EPS453	*Prunus persica*	INTEA-EPS
Pseudomonas fluorescens EPS82	*Pyrus communis*	INTEA-EPS
P. savastanoi 1628	*Olea europaea*	IVIA
P. savastanoi 1670	*Olea europaea*	IVIA
P. syringae pv. *phaseolicola* 6721 (CECT 321)	*Phaseolus vulgaris*	IVIA
P. syringae pv. *syringae* EPS94	*Pyrus communis*	INTEA-EPS
P. syringae pv. *tomato* 951-4	*Lycopersicon esculentum*	IVIA
P. syringae pv. *tomato* 984-3b	*Lycopersicon esculentum*	IVIA
Serratia marcescens EPS318	*Vitis vinifera*	INTEA-EPS
Xanthomonas arboricola pv. *juglandis* 1317.3df	*Juglans regia*	IVIA
X. vesicatoria 1779-2	*Capsicum annuum*	IVIA
X. vesicatoria 2133-2	*Capsicum annuum*	IVIA

[a]INTEA-EPS: Institut de Tecnologia Agroalimentària, Escola Politècnica Superior, Universitat de Girona, Girona, Spain. IVIA: M.M. López, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain.

2.4.2. Products

Benzothiadiazole, fosetyl-Al, ethephon, copper sulfate, streptomycin and kasugamycin were tested for their antibacterial *in vitro* activity. Stock solutions of chemical compounds were made up in distilled water, and added to molten CYE agar at 50 ºC on Petri plates to achieve concentrations of 634, 317, 158.5, 79.3, 39.6, 19.8, 9.9, 4.9, 2.5, 1.2 and 0.6 μg HPO_3^{2-} mL^{-1} for phosphonates (fosetyl-Al and ethephon). Benzothiadiazole and copper sulfate doses tested were 100, 50, 25, 12.5, 6.3, 3.1 and 1.6 μg a.i. mL^{-1}, and antibiotic (streptomycin and kasugamycin) concentrations were 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 μg a.i. mL^{-1}. Non-amended CYE was used as a control.

2.4.3. Assessment of *in vitro* activity and experimental design

Colony growth 5 days after bacteria inoculation was taken as tolerance to the corresponding dose of the chemical. An inhibitory effect of compounds was considered when growth of bacteria was inhibited.

The experimental design consisted of 14 strains, 6 products, and 7 to 11 doses (depending on product) with three replicates per treatment. The experiment was conducted twice.

3. RESULTS

3.1. EFFICACY OF COPPER DERIVATIVES IN FIRE BLIGHT CONTROL

3.1.1. Copper derivatives

The efficacy of copper derivatives in *E. amylovora* control in apple and pear was tested in potted plants and in flowers on detached branches. In plants, the five copper derivatives (Bordeaux mixture at 20% of metallic copper, Bordeaux mixture at 25%, copper hydroxide at 50%, copper oxide at 50% and copper oxychloride at 50%) were compared at two doses, 1.5 and 3 g Cu L^{-1}, and sprayed following three different strategies: preventive, protective and curative, as described previously (p. 55). Among the copper derivatives, the most effective were Bordeaux mixture 25% and copper hydroxide. In most cases, the lower dose of copper tested (1.5 g Cu L^{-1}) slowly reduced the disease severity, as was expected, while the highest dose (3 g Cu L^{-1}) was more effective in disease control. However, some exceptions were observed, such as copper oxychloride applied in curative strategy in pear or copper hydroxide in the curative strategy in apple. Surprisingly, the preventive strategy was the less effective in both species; mean disease severity was 24.3% in pear and 36.7% in apple (Figure 2.2.A and D). The protective strategy (copper sprays performed after lesion occurred in leaves and before bacterial inoculation) was more effective in pear (3.2%) than in apple (16.5%), and the curative strategy was slightly less effective in pear (13.2%) than in apple (8.6%) (Figures 2.2.B, C, E and F). In fact, each strategy had different effectiveness depending on product and host specie. Disease severity was higher in apple than in pear, mainly in preventive and protective strategies, whereas in curative strategy it was similar. The most effective products were the antibiotics. Between the two antibiotics, kasugamycin gave a better control of disease in all strategies and in both species. Streptomycin was also effective in comparison with copper compounds, but showed a slightly lower effect in preventive strategy on apple. ANOVA indicated a significant effect of product (P<0.001: R^2=0.73 in pear, and P<0.001: R^2=0.76 in apple), copper dose (P<0.007 in pear and P<0.001 in apple) and strategy used (P<0.001).

The efficacy of copper derivatives in fire blight control in pear and apple was also tested on flowers of detached branches (Figure 2.3). In flowers, five copper derivatives (Bordeaux mixture at 20% of metallic copper, Bordeaux mixture at 25%, copper hydroxide at 50%, copper oxide at 50% and copper oxychloride at 50%) were also compared at two doses, 1.5 and 3 g Cu L^{-1}, and sprayed following two different strategies: copper sprays performed before pathogen inoculation (preventive) and copper sprays applied after inoculation (curative). Among copper derivatives only Bordeaux mixture 20% and copper oxychloride reduced disease severity to 45.7% and 40%, respectively, when applied after inoculation on pear flowers (Figure 2.3.B).

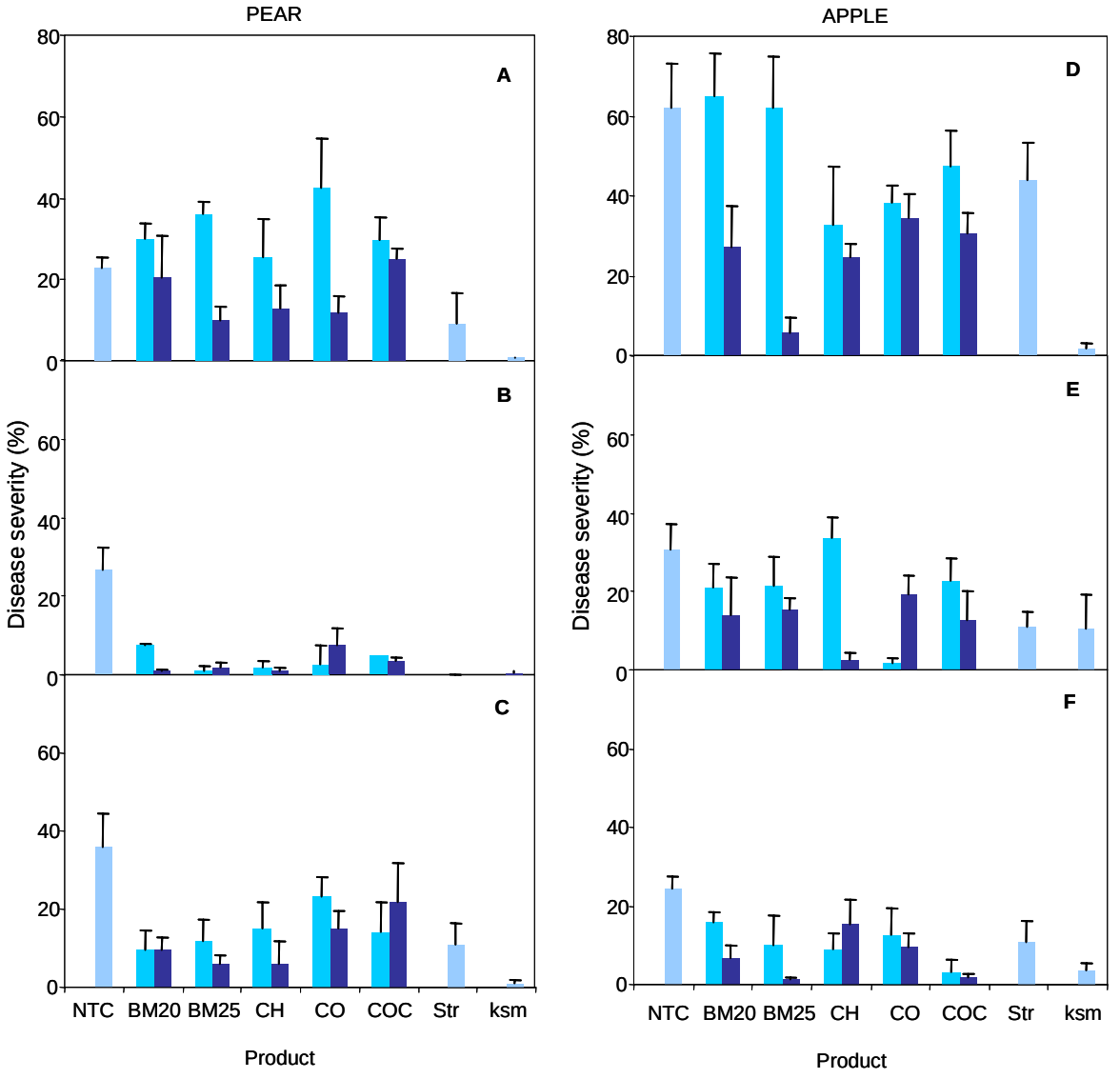


Figure 2.2. Effect of copper derivatives (BM20: Bordeaux mixture 20%; BM25: Bordeaux mixture 25%; CH: copper hydroxide; CO: copper oxide; COC: copper oxychloride) on disease severity in pear and apple plants inoculated with *E. amylovora* EPS101. Two doses of copper; 1.5 (■) and 3 (■) g Cu L^{-1} were evaluated for each product. Products were applied according to different strategies: before lesion occurred in leaves and before pathogen inoculation (preventive, **A and D**); after lesion in leaves and before inoculation (protective, **B and E**); and after lesion and pathogen inoculation (curative, **C and F**). Antibiotics (Str: streptomycin and ksm: kasugamycin) were applied at 100 mg a.i. L^{-1} and 30 mg a.i. L^{-1}, respectively, 2 h before bacteria inoculation. NTC: non-treated controls (■) consisted of pathogen inoculated plants without receiving sprays. Disease severity was scored 10 days after inoculation on pear and 15 days after inoculation on apple. Severity values correspond to the mean of three replicates. Error bars represent the standard error of the mean.

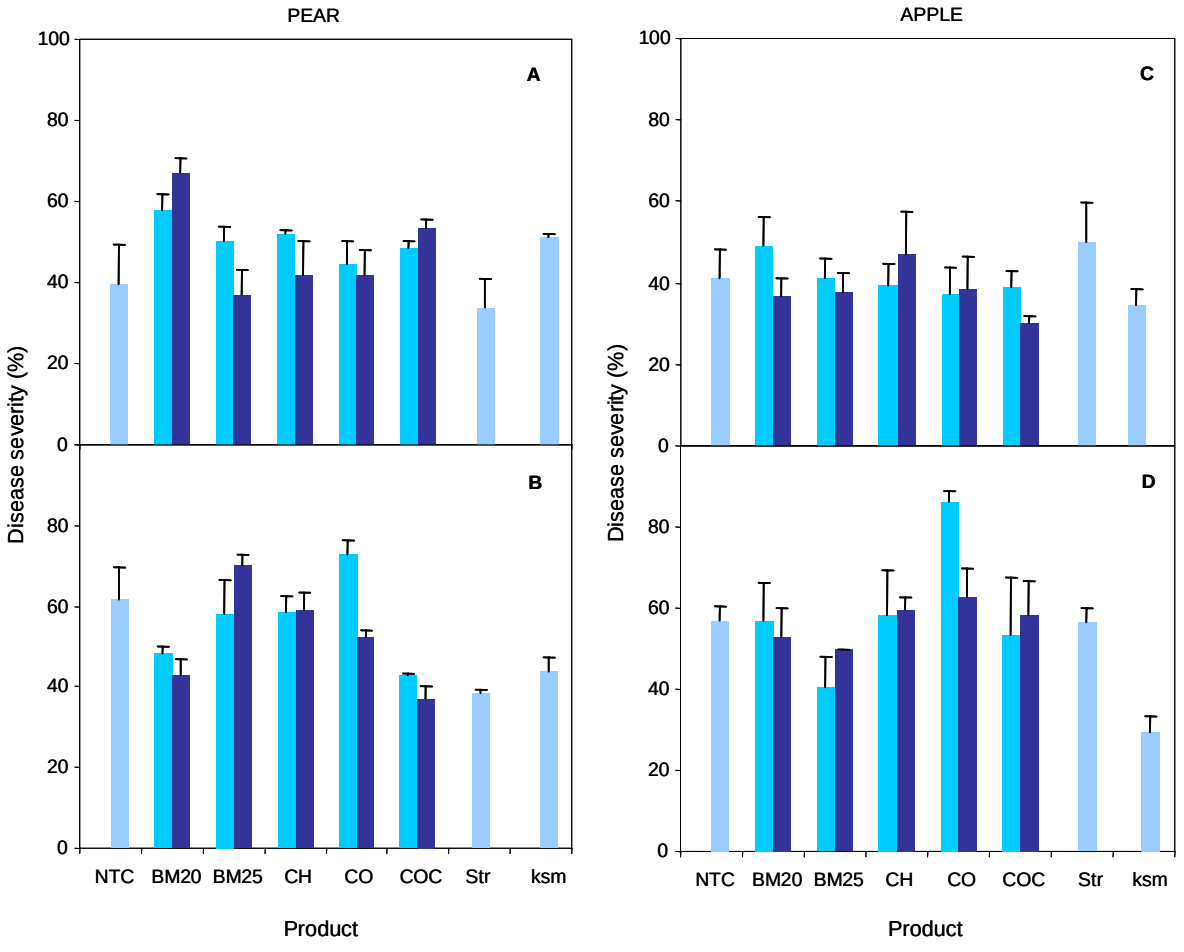


Figure 2.3. Effect of copper derivatives (BM20: Bordeaux mixture 20%; BM25: Bordeaux mixture 25%; CH: copper hydroxide; CO: copper oxide; COC: copper oxychloride) on disease severity in pear and apple flowers inoculated with *E. amylovora* EPS101. Two doses of copper; 1.5 (■) and 3 (■) g Cu L^{-1} were tested for each product. Products were applied according to different strategies: before pathogen inoculation (preventive, **A and C**) and after inoculation (curative, **B and D**). Antibiotics (Str: streptomycin and ksm: kasugamycin) were applied at 100 mg a.i. L^{-1} and 30 mg a.i. L^{-1}, respectively. NTC: non-treated controls (■). Disease severity was scored 7 days after inoculation on pear, and 12 days after inoculation on apple. Severity values correspond to the mean of three replicates. Error bars represent the standard error of the mean.

Bordeaux mixture 25% reduced disease severity to 45.1% on curative strategy in apple. Between the two doses of copper derivatives tested there were no differences. When products were sprayed before inoculation, differences among treatments were not observed, whereas when the products were sprayed after pathogen inoculation, a slight effect was observed in some treatments (Bordeaux mixture 25% on pear). Disease severity levels were no different between apple and pear flowers. Analysis of variance showed that no factor had significant effect. No significant effect of products (P=0.32: R^2=0.62 and P=0.26: R^2=0.56), dose of copper (P=0.20 and P=0.40), and strategies of spray (P=0.14 and P=0.05) on pear and apple, respectively, were observed. However, severity values on preventive sprays were slightly lower than those from curative sprays. In addition, no significant reduction of the disease was obtained with antibiotics, although, a slight reduction was observed in treatments with streptomycin compared with kasugamycin in pear and, conversely, in apple flowers the best antibiotic was kasugamycin.

3.1.2. Copper-mancozeb mixture

Efficacy of copper-mancozeb mixture in control of *E. amylovora* infection on pear and apple plants was evaluated. Copper sulfate 20% (2.5 g Cu L^{-1}) plus mancozeb (1.5 g L^{-1}) was sprayed onto plants either immediately after the mixture was made or 10 hours after its preparation (matured mixture). Products were applied according to preventive and protective strategies. In pear plants, analysis of variance with square root transformed values of disease severity indicated a significant effect of treatment (P<0.001: R^2=0.91), spraying strategy (P<0.001), and interaction between strategy and treatment on disease severity (P<0.001). The preventive strategy was more effective (83-98% of efficacy) than the protective strategy. All products applied in preventive strategy reduced significantly disease symptoms (60% reduction), in contrast with non-chemical-sprayed plants. No significant differences in disease severity were observed among products. Higher levels of infection in non-treated plants were obtained in pear (60%) than in apple plants (30%), so a more evident effect of different products was observed in pear (Figure 2.4). Preventive sprays of both matured and non-matured copper sulfate-mancozeb mixtures reduced disease severity in 80-90%. Similar reduction in severity was observed in preventive applications of copper sulfate alone. The highest reduction of infection (90%) was obtained in preventive sprays of matured copper-mancozeb mixture; this reduction was similar to that observed in kasugamycin sprayed pear plants. Protective sprays of matured and non-matured copper–mancozeb mixtures did not clearly reduce the disease severity in comparison with the non-treated control. However, protective application of copper sulfate alone, gave a significant reduction in disease severity (80% reduction). In apple, analysis of variance with square root transformed data showed that products had a significant effect (P<0.001: R^2=0.68), while the strategy (P=0.37), and the treatment-strategy interaction (P=0.64) had no significant effect on disease severity. Lower levels of disease severity were observed in apple plants sprayed with non-matured copper-mancozeb than in matured mixture sprayed ones. Similar infection levels were obtained when copper sulfate was sprayed

alone or mixed with mancozeb. The highest reduction in disease severity was obtained in plants sprayed with kasugamycin in both strategies for two plant species.

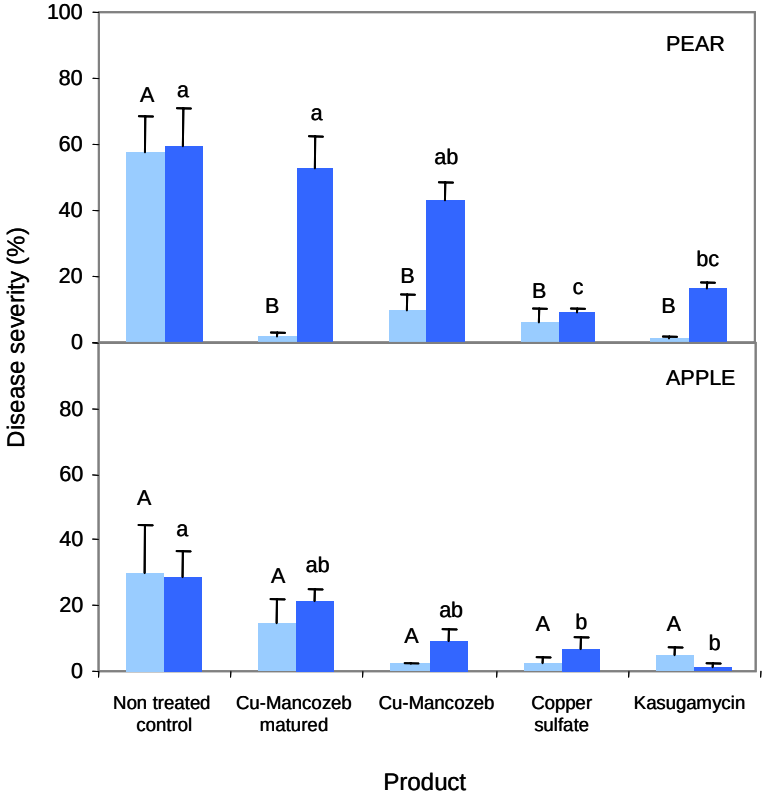


Figure 2.4. Effect of copper-mancozeb mixture on disease severity in pear and apple plants sprayed according to two different strategies; preventive (□) and protective (■) and inoculated with *E. amylovora* EPS101. Non-matured mixture (2.5 g Cu L^{-1} plus 1.5 g mancozeb L^{-1}) was prepared just before its application, while matured mixture was prepared 10 hours before spraying. Bordeaux mixture 20% 2.5 g Cu L^{-1} and kasugamycin 30 mg L^{-1} were also included as well as a non-treated control. Values are the mean of three replicates of three plants per treatment. Error bars indicate the standard error of the mean. Means with the same letter do not differ significantly (P=0.05) according to Tukey's mean separation test.

3.2. Efficacy of plant defense activators in control of fire blight

Two experiments were performed to compare the efficacy of three compounds which seem to act as plant defense activators: fosetyl-Al (1.86 g HPO_3^{2-} L^{-1}), ethephon (0.5 g HPO_3^{2-} L^{-1}), and benzothiadiazole (100 mg a.i. L^{-1}). Plants were sprayed with copper (1.5 g Cu L^{-1}) and antibiotics (streptomycin at 100 mg a.i. L^{-1} and kasugamycin at 30 mg a.i. L^{-1}) as reference compounds, and water as non-treated control. In the first experiment, two spray timings for fosetyl-Al and ethephon

were tested (1) 7 and 2 days before inoculation or (2) daily for the 5 days previous to pathogen inoculation. First experiment was repeated twice and no significant differences were observed between the two independent replicates (P=0.58: R^2=0.64), therefore data were pooled. There was a significant effect of products on disease severity (P<0.001: R^2=0.59). All products significantly reduced the disease severity, compared to the non-treated control, but Tukey's mean separation test (P=0.05) indicated no differences among products (Figure 2.6). The most effective products were ethephon and benzothiadiazole sprayed twice (7 and 2 days before inoculation) with 27 and 22.5% of disease severity, respectively. Phytotoxicity of phosphonates or benzothiadiazole was not observed in pear plants at dose used. However, ethephon produced some defoliation and inhibition of internodal elongation of plants when applied daily for the five days prior to inoculation (Figure 2.5).

In the second experiment, products were applied at the same rate and schedule as in the previous experiment, but phosphonates were only applied two times, 7 and 2 days, before inoculation. Similar levels of disease severity were obtained in non-treated plants and in copper and fosetyl-Al sprayed plants, so these products did not reduce the infection caused by *E. amylovora* on pear plants (Figure 2.7). Benzothiadiazole, streptomycin, ethephon and kasugamycin reduced to 40-80%, depending on product, the disease levels. Greatest reduction was obtained in ethephon and kasugamycin treated plants (75-80%), while benzothiadiazole and streptomycin reduced infection similarly (40-50%). Analysis of variance indicated a significant effect of products on disease severity (P<0.001: R^2=0.93).

Figure 2.5. Defoliation (DF, white arrows) in pear plants cv. Conference caused by ethephon 0.5 g HPO_3^{2-} L^{-1} sprayed during 5 days before *E. amylovora* inoculation.

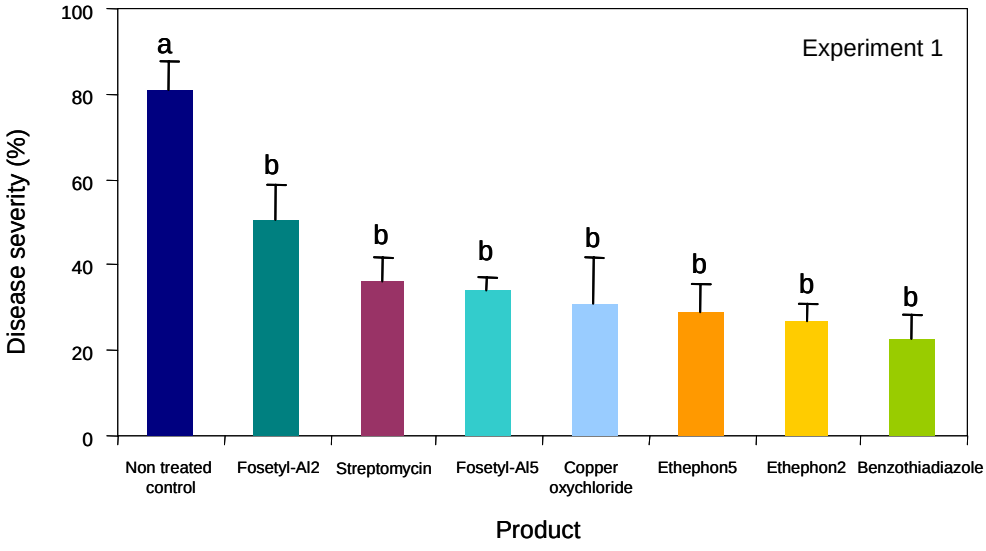


Figure 2.6. Disease severity in pear plants cv. Conference inoculated with *E. amylovora,* in non-treated control and after application of several products. Fosetyl-Al 1.86 g $HPO_3^{2-}L^{-1}$ (fosetyl-Al 2: applied twice, 7 and 2 days before inoculation; fosetyl-Al 5: applied 5 times, 5, 4, 3, 2 and 1 day before inoculation), ethephon 0.5 g $HPO_3^{2-}L^{-1}$ (ethephon 2: applied twice, 7 and 2 days before inoculation; and ethephon 5: applied 5 times, 5, 4, 3, 2 and 1 day before inoculation), benzothiadiazole 100 mg a.i. L^{-1}, copper oxychloride 1.5 g Cu L^{-1}, streptomycin 100 mg a.i. L^{-1} and kasugamycin 30 mg a.i. L^{-1}. Values are the mean of pooled data from two independent experiments with three repetitions of three plants each. Error bars indicate standard error of the mean. Means with the same letter do not differ significantly (P=0.05) according to Tukey's mean separation test.

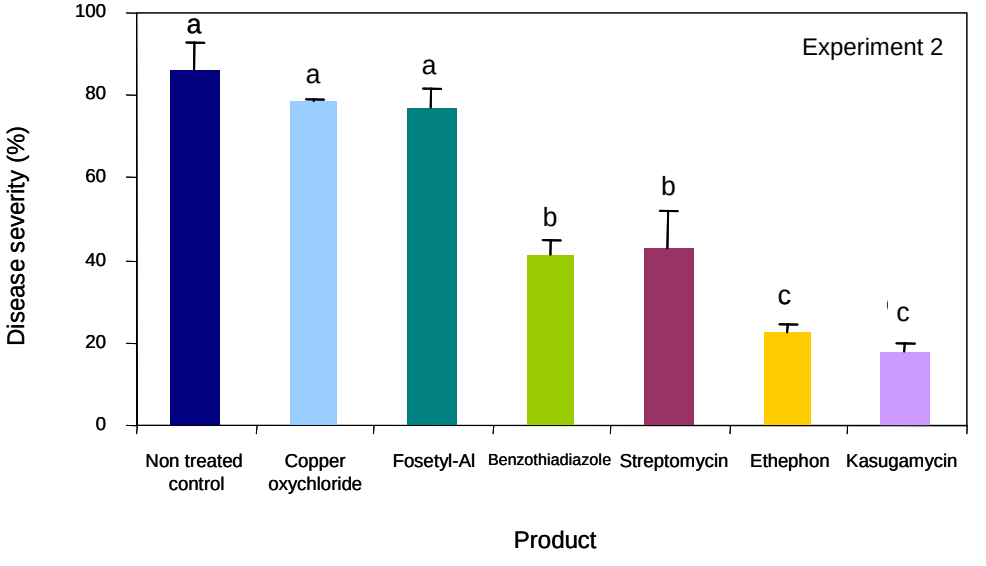


Figure 2.7. Disease severity in pear plants cv. Conference inoculated with *E. amylovora,* in non-treated control and after application of several products. Fosetyl-Al 1.86 g $HPO_3^{2-}L^{-1}$, ethephon 0.5 g $HPO_3^{2-}L^{-1}$, and benzothiadiazole 100 mg a.i. L^{-1}, applied twice (7 and 2 days before inoculation), copper oxychloride 1.5 g Cu L^{-1}, streptomycin 100 mg a.i. L^{-1} and kasugamycin 30 mg a.i. L^{-1} applied 2 hours before bacterial inoculation. Values are the mean of three replicates. Error bars indicate standard error of the mean. Means with the same letter do not differ significantly (P=0.05) according to Tukey's mean separation test.

3.2.1. Optimal spray timing and dose of phosphonate derivatives

The optimal spray timing was determined for fosetyl-Al and ethephon. Products were sprayed on pear plants from 7 to 1 day before bacteria inoculation. Products were applied once, at the rate of 1.86 g $HPO_3^{2-}L^{-1}$ for fosetyl-Al, and 0.5 g $HPO_3^{2-}L^{-1}$ for ethephon. ANOVA indicated a significant effect of spray timing on disease severity (P<0.001: R^2=0.54), but a non-significant effect of phosphonate (P=0.86), and their interaction (P=0.08). Statistical analysis was performed with square root transformed severity values. The best disease control was achieved when fosetyl-Al was applied 5 days before inoculation (35% disease reduction) and when ethephon was sprayed 7 days before inoculation (60% disease reduction) (Figure 2.8). Fosetyl-Al sprayed 6 or 7 days before bacteria inoculation reduced disease severity similarly than when it was sprayed 5 days before inoculation, therefore higher intervals between treatment and inoculation did not improve fosetyl-Al efficacy. An increased time interval between treatment with ethephon and bacteria inoculation was correlated with a reduction of disease severity levels. Although no significant differences were observed between products, higher reduction in severity was observed when ethephon was sprayed on plants 6 and 7 days before bacteria inoculation.

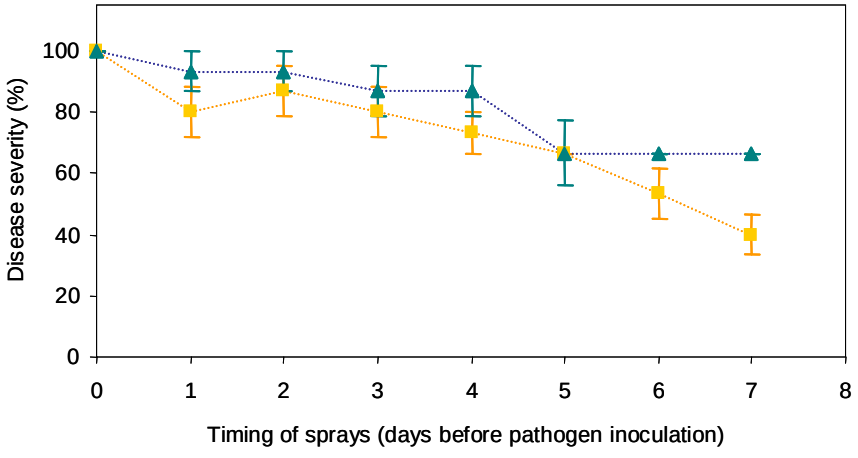


Figure 2.8. Disease severity in pear plants cv. Conference inoculated with *E. amylovora* after treatment with fosetyl-Al 1.86 g $HPO_3^{2-}L^{-1}$ (▲) or ethephon 0.5 g $HPO_3^{2-}L^{-1}$ (■) at different sprayed interval before pathogen inoculation. Values are the mean of five replicates. Error bars indicate the standard error of the mean.

To determine the optimal dose of fosetyl-Al, four doses were tested; 0.62, 0.93, 1.86 and 3.72 g $HPO_3^{2-}L^{-1}$, applied once, 7 days before bacteria inoculation. A significant effect of dose on disease severity was observed (P<0.001: R^2=0.85). Increased doses of fosetyl-Al directly decreased severity levels on pear plants (Figure 2.9). The highest reduction on severity (45%) was obtained at the maximal dose tested (3.72 g $HPO_3^{2-}L^{-1}$). No phytotoxic effects were observed in pear plants sprayed with fosetyl-Al at any dose. From severity values on each dose and replicate the median effective dose (ED_{50}) and 90% effective dose (ED_{90}) were calculated by means of linear regression. Fosetyl-Al ED_{50} and ED_{90} corresponded to 3.34 g $HPO_3^{2-}L^{-1}$ and 6.99 g $HPO_3^{2-}L^{-1}$, respectively (Table 2.10).

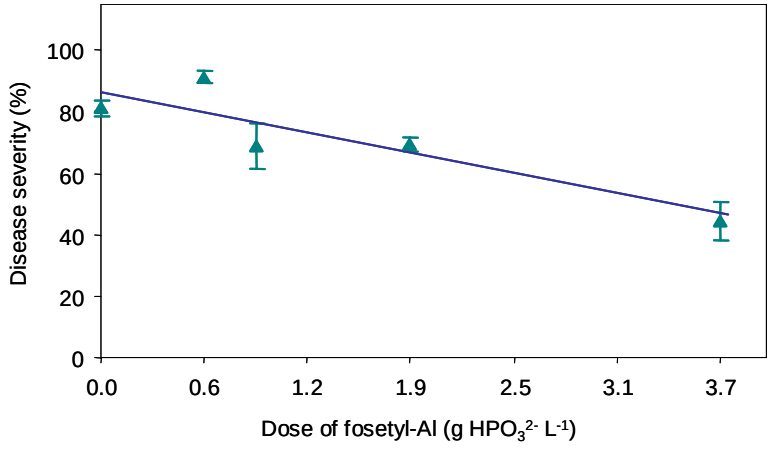


Figure 2.9. Effect of fosetyl-Al dose on disease severity in pear plants cv. Conference inoculated with *E. amylovora*. Fosetyl-Al was sprayed 7 days before bacteria inoculation. Solid blue line represents predicted values by linear regression. Values are the mean of three replicates of three plants. Error bars indicate standard error of the mean.

Table 2.10. Regression equation parameters, median effective dose (ED_{50}) and 90% effective dose (ED_{90}) of fosetyl-Al in pear plants cv. Conference inoculated with *E. amylovora*

Intercept (standard error)	**Slope** (standard error)	**R^2** [a]	***P*** [b]	**Effective Dose** (g $HPO_3^{2-}L^{-1}$)[c]	
				ED_{50}	**ED_{90}**
86.67 (5.80)	-10.97 (3.01)	0.82	0.036	3.34 (0.43)	6.99 (1.35)

[a] R^2 of the adjustment of predicted values by the linear model with respect to the observed ones
[b] Probability of the linear model
[c] Value calculated from the linear function obtained by simple regression of disease severity values through the fosetyl-Al dose (Figure 2.9)

3.2.2. Optimal benzothiadiazole spray timing and dose

To determine the optimal timing of benzothiadiazole (100 mg a.i. L^{-1}), pear plants were sprayed at different time intervals, before bacteria inoculation. The experiment was carried out twice. Analysis of variance with square root transformed disease severity values indicated no highly significant differences between the two independent experiments (P=0.05: R^2=0.57), therefore data were pooled. A significant effect of time interval on disease severity was observed (P<0.001: R^2=0.72).

An increase in time interval between benzothiadiazole spray and pathogen inoculation was correlated with a reduction on disease severity (Figure 2.10). Time interval higher than 7 days did not increase benzothiadiazole efficacy. Significant reduction in disease severity (40-60%) was obtained when benzothiadaizole was sprayed 6-7 days before bacteria inoculation.

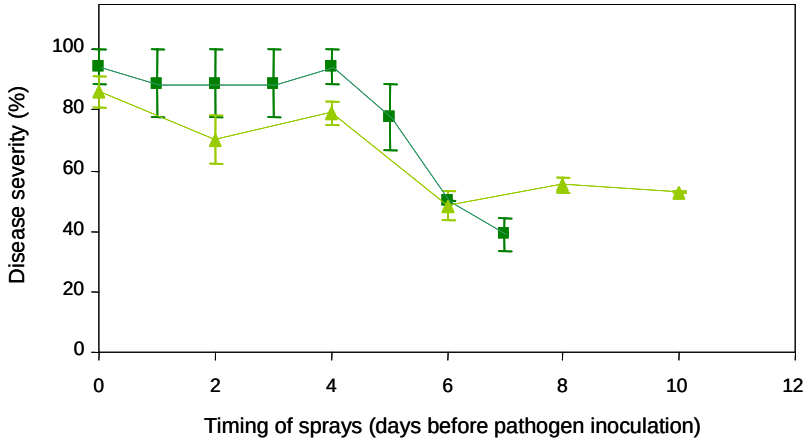


Figure 2.10. Effect of benzothiadiazole spraying time on disease severity in pear plants cv. Conference inoculated with *E. amylovora*. Benzothiadiazole (100 mg a.i. L^{-1}) was sprayed at different time interval before bacteria inoculation. Two independent experiments were performed (experiment 1■ and 2▲). Values are the mean of three replicates of three plants. Error bars indicate standard error of mean.

Two experiments were carried out to determine the optimal dose of benzothiadiazole for fire blight control. Eight doses of benzothiadiazole from 0 to 250 mg a.i. L^{-1} were tested. Analysis of variance indicated no significant differences between the two independent experiments performed (P=0.06: R^2=0.72), so data were pooled. A significant effect of dose of benzothiadiazole on disease severity was obtained (P<0.001: R^2=0.70). A progressive reduction in disease severity was observed when benzothiadiazole dose increased from 0 to 150 mg a.i. L^{-1}; however, doses higher than 150 mg a.i. L^{-1} did not improve its efficacy in disease control (Figure 2.11). Therefore, different patterns of dose effect on disease severity were observed depending on the dose range. Doses from 0 to 75 mg a.i. L^{-1} produced a weak reduction of disease severity, whereas 75 to 150 mg a.i. L^{-1} provoked a more intense reduction in *E. amylovora* infection, and finally, benzothiadiazole doses higher than 150 mg

a.i. L^{-1} gave a constant reduction of disease regardless of dose. Maximal disease reduction obtained was 50% when applied at 150 mg a.i. L^{-1}. Regression of disease severity on dose of benzothiadiazole allowed us to calculate the median effective dose (ED_{50}) and 90% effective dose (ED_{90}) (Table 2.11). ED_{50} was around 182.93 mg a.i. L^{-1} and ED_{90} was 343.48 mg a.i. L^{-1}. There was a threshold in effectiveness, from which increased benzothiadiazole dose did not improve the disease control.

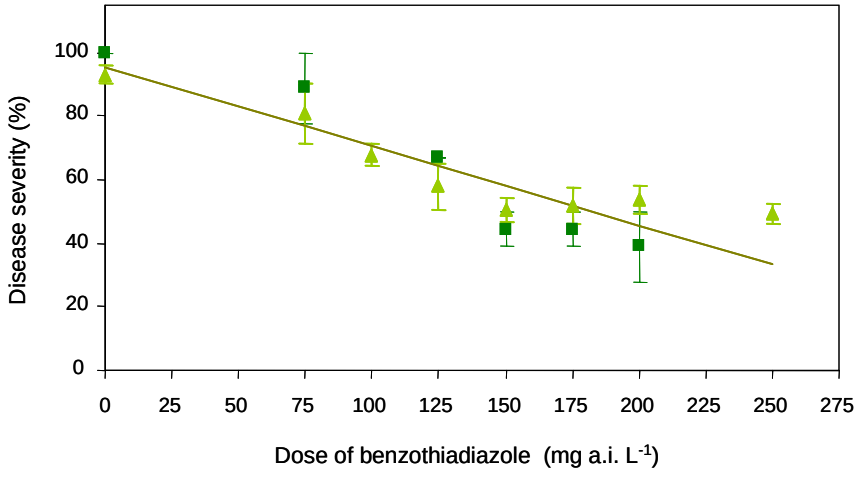


Figure 2.11. Effect of benzothiadiazole dose on disease severity in pear plants cv. Conference inoculated with *E. amylovora*. Benzothiadiazole was sprayed 7 days before pathogen inoculation. Two independent experiments were performed (■ experiment 1, ▲ experiment 2). Solid green line represents the predicted values obtained by linear regression. Values are the mean of three replicates. Error bars indicate standard error of the mean.

Table 2.11. Regression equation parameters, median effective dose (ED_{50}) and 90% effective dose (ED_{90}) of benzothiadiazole in pear plants cv. Conference inoculated with *E. amylovora*

Intercept (standard error)	**Slope** (standard error)	$R^{2\,a}$	P^{b}	**Effective Dose** (mg a.i. L^{-1})^{c}	
				ED_{50}	**ED_{90}**
95.58 (3.71)	-0.25 (0.03)	0.70	<.0001	182.93 (6.67)	343.48 (10.61)

[a] R^2 of the adjustment of predicted values by the linear model with respect to the observed ones
[b] Probability of the linear model
[c] Value calculated from the linear function obtained by simple regression of disease severity values through the benzothiadiazole dose

3.3. Efficacy of plant defense activators combined with copper and antibiotics in fire blight control

Experiments where performed under controlled environment conditions, fosetyl-Al (1.86 g $HPO_3^{2-}L^{-1}$) and benzothiadiazole (100 mg a.i. L^{-1}) were sprayed alone, mixed or combined with half dose of copper sulfate, streptomycin and kasugamycin on pear plants indicated a significant effect of product on disease severity (P<0.001: R^2>0.80). Differences in reduction of disease severity were obtained on mixed and combined sprays (Table 2.12). A significant reduction (50%) in disease levels was observed in benzothiadiazole-kasugamycin and benzothiadiazole-streptomycin mixtures, with infection levels similar to those from streptomycin. Fosetyl-Al and fosetyl-Al-antibiotic mixtures did not reduce significantly the disease, whereas combined sprays of fosetyl-Al and antibiotics reduced disease levels in 80-90% in reference to non-treated plants. Copper sulfate alone and mixed with fosetyl-Al or with benzothiadiazole reduced significantly but moderately the disease severity (29%). Moreover, when copper was applied in combined strategy with fosetyl-Al or with benzothiadiazole, its efficacy increased and a significant reduction in disease severity was obtained (60-80%). Combined treatment of fosetyl-Al and copper had some phytotoxic effect on pear plants leaves, evident in necrotic spots (Figure 2.12). Combined strategy gave a higher reduction in disease levels for all products than the mixed one. Benzothiadiazole-antibiotics and fosetyl-Al-kasugamycin combined sprays produced a reduction in disease levels of 90% and the best results in *E. amylovora* control.

In experiments performed under greenhouse conditions, at Angers (France) the effectiveness of combined sprays of benzothiadiazole with kasugamycin was tested on pear and apple seedlings. Additionally a biocontrol experimental agent was included. Analysis of variance indicated a significant effect of products (alone or combined) on disease severity (P<0.001: R^2=0.79), while no significant effect of plant species (P=0.29) nor of the interaction (P=0.12) was observed. All products reduced significantly the disease severity in comparison with that from non-treated plants (Table 2.13). The best fire blight control was obtained in plants sprayed with kasugamycin and in benzothiadiazole-kasugamycin combined sprays. Benzothiadiazole and biocontrol agent sprayed separately showed similar efficacy in disease control, in pear and apple, and when they were combined a higher reduction of disease was observed on pear seedlings. Fosetyl-Al was the less effective treatment and reduced disease severity by 20-30%. Benzothiadiazole was more effective than fosetyl-Al, and when combined with the biocontrol agent significantly reduced disease severity in pear (5-17% depending on data assessment), while in apple no differences were found. Disease assessment was performed 7 and 14 days after bacteria inoculation, and higher levels of disease were obtained 14 days after inoculation.

In the field experiment analysis of variance indicated a significant effect of treatments on disease incidence (P<0.001: R^2=0.70). Significant differences among products were observed (Table 2.14). A significant control of blossom blight of apple was recorded, when fosetyl-Al, benzothiadiazole, copper, the biocontrol agent and kasugamycin were applied. Products sprayed alone were more

effective than when combined with benzothiadiazole. Combined sprays of benzothiadiazole-kasugamycin did not differ significantly from kasugamycin sprays. Benzothiadiazole-biocontrol agent sprays did not reduce disease incidence compared to non-treated plants.

Figure 2.12. Phytotoxic effect in pear plants cv. Conference (white arrows) of fosetyl-Al (1.86 g $HPO_3^{2-}L^{-1}$)-copper sulfate (0.37 g Cu L^{-1}) mixture sprayed 7 days before *E. amylovora* inoculation. NS: Necrotic spot.

Table 2.12. Effect of products simultaneously sprayed (mixed strategy) or consecutively sprayed (combined strategy) on disease severity (%) of pear plants cv. Conference inoculated with *E. amylovora* EPS101 under controlled environment conditions

	Strategy of application[b]			
Product[a]	**Simultaneous sprays**[c]		**Consecutive sprays**	
Non-treated control	76.1	a	94.0	a
Fosetyl-Al (1.86 g $HPO_3^{2-}L^{-1}$)	70.7	ab	26.8	bcd
Fosetyl-Al + Kasugamycin	67.9	ab	2.6	e
Fosetyl-Al + Streptomycin	59.3	abc	18.3	bcde
Benzothiadiazole + Copper sulfate	54.6	abc	34.3	b
Copper sulfate (1.5 g Cu L^{-1})	54.4	abc	89.1	a
Fosetyl-Al + Copper sulfate	54.0	abc	18.1	bcde
Benzothiadiazole (100 mg a.i. L^{-1})	42.9	bcd	28.4	bc
Benzothiadiazole + Kasugamycin	36.0	cd	4.5	de
Benzothiadiazole + Streptomycin	35.2	cd	5.6	cde
Streptomycin (100 mg a.i. L^{-1})	31.5	cd	12.1	bcde
Kasugamycin (30 mg a.i. L^{-1})	11.6	d	1.8	e
Mean standard error	*5.73*		*3.75*	

[a] Chemicals were sprayed alone, mixed or combined. When combined or mixed, copper sulfate and antibiotics doses were a half than when they were sprayed alone
[b] Severity values are the mean of three replicates of three plants
[c] Mixed products were added to an aqueous solution and the mixture was sprayed to plants 7 days before bacteria inoculation. Combined products were sprayed separately to plants (benzothiadiazole and fosetyl-Al, 7 days before inoculation, and the others 2 h before bacteria inoculation). Means within the same column followed by the same letter did not differ significantly (P=0.05) according to Tukey's mean separation test

Table 2.13. Disease severity (%) in pear cv. Kirchensaller and apple cv. Golden Delicious seedlings sprayed with products and inoculated with *E. amylovora* CFBP1430, under greenhouse conditions

Product[a]	Plant species[b]							
	pear				apple			
	7 days[c]		14 days		7 days		14 days	
Non-treated control	44.2	a	69.2	a	65.8	a	78.3	a
Fosetyl-Al (1.86 g HPO_3^{2-} L^{-1})	16.7	ab	47.5	ab	35.0	b	55.0	ab
Benzothiadiazole (150 mg a.i. L^{-1})	17.5	ab	40.0	ab	10.8	bc	30.0	bc
Biocontrol agent ($7x10^8$ cfu mL^{-1})	15.0	ab	31.7	ab	11.7	bc	36.7	bc
Copper sulfate (1.5 g Cu L^{-1})	7.5	ab	21.7	b	21.7	bc	35.0	bc
Benzothiadiazole + biocontrol agent	5.0	b	16.7	b	25.0	bc	51.7	ab
Benzothiadiazole + kasugamycin	2.5	b	15.0	b	2.5	c	5.8	c
Kasugamycin (40 mg a.i. L^{-1})	2.5	b	12.8	b	1.7	c	8.3	c
Mean standard error	*5.75*		*7.86*		*5.25*		*6.24*	

[a] Chemicals were applied alone or consecutively (combined strategy). Plant defense inducers (fosetyl-Al and benzothiadiazole) were applied 7 and 4 days before bacteria inoculation. Copper, antibiotics and the biological agent were sprayed 2 h before inoculation
[b] Severity values are the mean of ten replicates
[c] Date of disease severity assessment (days after inoculation). Means within the same column followed by the same letter do not differ significantly (P=0.05) according to Tukey's mean separation test

Table 2.14. Disease incidence in apple trees sprayed with chemicals and inoculated with *E. amylovora* CFBP1430

Product[a]	Disease incidence (%)[b]	
Non-treated control	58.0	a
Biocontrol agent ($7x10^8$ cfu mL^{-1})	42.1	ab
Benzothiadiazole + biocontrol agent	27.7	abc
Benzothiadiazole + kasugamycin	26.1	abc
Kasugamycin (40 mg a.i. L^{-1})	19.3	bc
Fosetyl-Al (1.86 g HPO_3^{2-} L^{-1})	12.5	bc
Copper sulfate (1.5 g Cu L^{-1})	10.9	bc
Benzothiadiazole (150 mg a.i. L^{-1})	3.7	c
Mean of standard error	*6.08*	

[a] Chemicals were applied alone or consecutively (combined strategy)
[b] Incidence values are the mean of three replicates
Means within the same column followed by the same letter do not differ significantly (P=0.05) according to Tukey's mean separation test. Disease incidence was assessed 21 days after pathogen inoculation.

3.4. *IN VITRO* ANTIBACTERIAL ACTIVITY OF CHEMICALS

Chemicals were evaluated for *in vitro* activity against 14 strains of plant-pathogenic and non-plant pathogenic bacteria. Inhibition of colony development on low complexing CYE agar amended with compounds is shown in Table 2.15. Inhibition of colony growth in CYE agar by benzothiadiazole and phosphonate derivatives (fosetyl-Al and ethephon) was very low. The minimal inhibitory concentration (MIC) was higher than 100 μg a.i. mL^{-1} for benzothiadiazole against all strains tested and between 317 and 634 μg HPO_3^{2-} mL^{-1} for phosphonates in most of the strains. Only copper and antibiotics showed antibacterial activity *in vitro* (MIC ≤ 4-50 μg a.i. mL^{-1}). *E. amylovora* EPS101 had low sensibility to plant defense inducers, a moderate sensibility to copper and streptomycin and was highly sensible to kasugamycin compared with the other bacterial strains.

Table 2.15. Minimal inhibitory concentrations of benzothiadiazole, fosetyl-Al, ethephon, copper sulfate, streptomycin and kasugamycin for several bacterial strains on amended CYE agar

Bacteria	Product[a]					
	BTH	**Fos**	**Et**	**Cu**	**Str**	**Ksm**
Erwinia amylovora EPS101	>100[b]	317-634	317-634	25-50	64-128	8-16
E. herbicola EPS21	>100	>634 [b]	317-634	25-50	64-128	8-16
E. herbicola EPS453	>100	317-634	317-634	25-50	32-64	8-16
Pseudomonas fluorescens EPS82	>100	317-634	317-634	50-100	16-32	16-32
P. savastanoi 1628	>100	317-634	317-634	12.5-25	16-32	<4
P. savastanoi 1670	>100	317-634	317-634	12.5-25	16-32	<4
P. syringae pv. *syringae* EPS94	>100	317-634	317-634	50-100	16-32	<4
P. syringae pv. *phaseolicola* 6721	>100	158-317	158-317	6-12	16-32	<4
P. syringae pv. *tomato* 951-4	>100	317-634	317-634	50-100	16-32	<4
P. syringae pv. *tomato* 984-3b	>100	317-634	317-634	25-50	16-32	<4
Serratia marcescens EPS318	>100	317-634	317-634	25-50	128-256	32-64
Xanthomonas vesicatoria 1779-2	>100	317-634	317-634	12-25	32-64	32-64
X. vesicatoria 2133-2	>100	317-634	158-317	12-25	32-64	32-64
X. arboricola pv. *juglandis* 1317.3df	>100	158-317	158-317	12-25	16-32	32-64

[a] BTH: benzothiadiazole; Fos: fosetyl-Al; Et: ethephon; Cu: copper sulfate; Str: streptomycin; Ksm: kasugamycin. Phosphonate concentration is expressed as μg HPO_3^{2-} mL^{-1}; concentration of antibiotics, copper and benzothiadiazole is expressed as μg a.i. mL^{-1}

[b] MIC for the highest dose tested

4. DISCUSSION

Fire blight caused by *E. amylovora* is currently controlled by multiple applications of copper or antibiotics. However there are not many reports about the effectiveness of different copper formulations in fire blight control. In this work we have demonstrated that the effectiveness of copper derivatives depends on the dose, the strategy of spraying (preventive, protective or curative) and the plant material used (pear or apple, plants or flowers). In assays performed on pear and apple plants, the best efficacy of copper derivatives was achieved with a protective strategy, when leaf wounds were protected with copper before pathogen inoculation. This strategy could be very important in orchard conditions after real risk situations such as hail and frost. The efficacy of the curative strategy was also especially good at the highest dose of copper (3 g Cu L^{-1}), although a curative activity of bactericides is rare. This agrees with other published studies in which bactericides were effective against *E. amylovora* only applied prior to, or up to 24 hours after inoculation (Brisset *et al*., 1990). Surprisingly, the lowest efficacy was obtained in the preventive strategy, when copper derivatives were sprayed before leaf wounding and pathogen inoculation. This was probably due to the methodology used, because in the preventive strategy coppers were sprayed first, then a wound was made in the leaf and finally the pathogen was inoculated; therefore when the wound was made it was not completely protected by copper against later pathogen inoculation, and the time between wounding and pathogen inoculation was short (2 hours). In curative strategy, copper was sprayed after wounding and inoculation of pathogen, so the time between wounding and inoculation was also short (2 hours) but as copper was sprayed after bacterial inoculation the amount of inoculum was probably highly reduced than in the preventive strategy. Finally, in protective strategy copper was sprayed after wounding and before pathogen inoculation; this was the most effective strategy for two reasons; copper protected the wound, and the time between wounding and bacteria inoculation was higher (4 hours). Crosse *et al*. (1972) demonstrated that high time interval between wound and inoculation reduces more effectively disease severity than short intervals. The variability in the efficacy of products could be due to the experimental procedure in which a wound on the leaves is necessary to develop *E. amylovora* infection and to the bacterial inoculation method used, because the amount of pathogen inoculum was not probably always the same. Probably, if the inoculation methods were more precise and always the same amount of pathogen was inoculated, the results from the different experiments would be more consistent. Among the most effective copper compounds we found Bordeaux mixture 25% and copper hydroxide. In plants the median effectiveness of Bordeaux mixture 25% (3 g Cu L^{-1}) was 78 % (50-94%), and of copper hydroxide (3 g Cu L^{-1}) was 67% (37-97%), in comparison with the kasugamycin with a median effectiveness of 90% (66-98%). In assays performed on flowers of detached branches differences in efficacy were not observed among copper derivatives, probably due to the fact that the inoculation method was very efficient in introducing the pathogen into the flower tissues, and that the flowers of detached branches

displayed less defenses in comparison with the whole plant. Although artificial bacteria inoculations succeeded in developing infections in plants or branches, artificial inoculation does not always imitate natural infections adequately. Further experiments (probably at a lower dose of pathogen) should be tested since inoculations and sprays on detached flowers failed to test the effectiveness of compounds on fire blight control, and this method was discarded for next experiments. However, in countries like Spain this is the only method available for testing the efficacy of chemicals in flowers. It must be also taken into account that some phytotoxic effects were observed with copper oxide in apple, and Bordeaux mixture 20% in pear flowers.

Differences among copper compounds in their efficacy in fire blight control, although sprayed at the same copper dose, may be due to the disparity in the dissolving capacity of the compounds in water, in particle size or in adhesion capacity. In spite of the procedure used on flower assays, our results indicated that no differences were found among copper compounds and are in agreement with those of Kooistra and Gruyter (1984) who found that copper sulfate and copper oxychloride are equally effective against flower infection provided that the amount of copper applied is the same. However, in field experiments, copper sulfate was the most efficient among other copper salts (Paulin and Lachaud, 1984). Bordeaux mixture was not significantly more effective than copper hydroxide or copper oxychloride on apple trees in an orchard (Burr and Norelli, 1984). Saygili and Üstün (1996) investigated the efficacy of some copper compounds *in vitro* and on pear fruitlets cv. Williams, and they obtained that copper sulfate showed lower activity when compared with copper hydroxide and copper oxychloride. All these results are consistent with ours and indicate that the effectiveness of copper derivatives varies according to experimental conditions or plant material used. Alternative products for chemical control of *E. amylovora* need to be explored since the occurrence of spontaneous and low-level copper tolerance among some strains of *E. amylovora* may indicate a potential for the development of resistance to copper compounds (Loper *et al*, 1991).

The efficacy of two antibiotics (streptomycin sulfate and kasugamycin) in *E. amylovora* infection control was also tested in these experiments. In certain cases kasugamycin seemed to be more effective than streptomycin, mainly in preventive and curative strategies and on apple more than pear. These results correlated well with those obtained by Kooistra and Gruyter (1984) who found that under artificial conditions the curative action of kasugamycin seemed to be better than that of streptomycin. Aldwinckle and Norelli (1990) also tested the efficacy of kasugamycin in apple trees in an orchard trial with artificial bacteria inoculations, and concluded that kasugamycin significantly reduced the proportion of blossom clusters infected when compared with a water check, and was significantly more effective in control of blossom blight than streptomycin 100 mg a.i. L^{-1}. However higher doses of kasugamycin tested (600, 300, 150 mg a.i. L^{-1}) were clearly phytotoxic, causing petal burn, foliar damage, and poor fruit set. While lower rates of kasugamycin (150 or 50 mg a.i. L^{-1}) were not significantly different in disease control than streptomycin, and no serious phytotoxicity problems were observed.

It has been demonstrated that copper compounds, combined with dithiocarbamates show satisfactory efficacy in fire blight control, under *in vitro* test (Saygili and Üstün, 1996), and under artificial and natural flower infection (kooistra and Gruyter, 1984). In our experiments copper-mancozeb mixture applied on pear in a preventive strategy did not improve the efficacy of copper solution, with a reduction of *E. amylovora* infection similar to kasugamycin. Additionally no differences on efficacy were observed between matured and non-matured copper-mancozeb mixtures. However, in the protective strategy only copper solution was significantly effective in bacterial infection control in comparison with the two copper-mancozeb mixtures. On apple, differences among treatments were not so noticeable but copper solution also showed the best efficacy. As our objective was to improve the copper efficacy we did not test mancozeb alone. In conclusion, only in pear a preventive spray of copper-mancozeb mixture give good efficacy in *E. amylovora* infection control, but without improving the copper efficacy. In these experiments preventive sprays were more effective in reducing the disease levels than protective sprays.

Under controlled environment conditions, application of plant defense inducers (phosphonate derivatives and benzothiadiazole) reduced significantly the disease severity compared with the non-treated control. In preliminary experiments fosetyl-Al, ethephon and benzothiadiazole sprayed 7 and 2 days before bacteria inoculation at standard rates (1.86, 0.5 g HPO_3^{2-} L^{-1} and 100 mg a.i. L^{-1}, respectively) showed similar efficacy in disease control than streptomycin, and better than copper. We observed that the effectiveness of phosphonates and benzothiadiazole was dependent on spray time and dose. The best control of fire blight was achieved when fosetyl-Al was sprayed 5 days before pathogen inoculation, increased time intervals did not improve the disease control. Conversely to results obtained by Larue and Gauliard (1993) who found that as nearest was the application of fosetyl-Al to the infection, better was the control of fire blight. Multiple applications of fosetyl-Al before bacteria inoculation were not tested because Chase (1993) demonstrated that on bacterial diseases multiple applications of fosetyl-Al before inoculation sometimes gave better control but frequently resulted in increased disease severity or did not differ significantly from a single preinoculation application. The effectiveness of fosetyl-Al increased with higher doses although differences among doses were not very significant. The high ED_{50} obtained for fosetyl-Al (3.34 g HPO_3^{2-} L^{-1}) indicates a weak *in vivo* activity of this chemical against *E. amylovora* on pear by foliar application. Our results contrasted with data reported by Chase (1993) indicating that bacterial disease control on several ornamental plants was more or less independent of the rate of fosetyl-Al within the range from 0.96 to 4.8 mg a.i. L^{-1}. But, they are in agreement with those from Moragrega *et al*. (1998) showing a significant dose-response relationship in control of bacterial blast of pear. In orchard trials fosetyl-Al (2.24-4.48 kg a.i. ha^{-1} at 24, 96 or 120 hours prior to inoculation) did not significantly reduce the amount of blossom blight in comparison with untreated controls and was less effective than streptomycin (Norelli and Aldwinckle, 1993).

Ethephon gave the best control of fire blight when sprayed 7 days before bacteria inoculation. It is remarkable the fact that some defoliation was observed in plants when multiple preinoculation applications were performed, as related Moragrega *et al*. (1998).

Control of *E. amylovora* infection with benzothiadiazole 100 mg a.i. L^{-1} ranged from 40 to 60% in whole plant experiments performed under controlled environment conditions. This efficacy was comparable to that previously reported by other authors; 30-80% in orchard trials (Thomson *et al*., 1998), 69% in scions in the greenhouse, and 50% in trees in orchard (Brisset *et al.,* 2000). The benzothiadiazole efficacy increased with longer preinoculation time interval. The best time interval between product spray and pathogen inoculation was 7 days, this is in accordance with observations by Tally *et al*. (1999) indicating that for maximum efficacy of BTH an interval between leaf treatment and exposure to the pathogen was required. The effectiveness of benzothiadiazole is also affected by dose; the maximal disease reduction was obtained with 150 mg a.i. L^{-1} and higher doses did not improve its efficacy. It is probably due to the mechanism of action of BTH, as suggested Tally *et al*. (1999) it appears that once the plant defenses are induced by BTH, higher rates of application do not improve disease protection or residual activity. Our results disagree with those of Schweizer *et al*. (1999) who found an inverse correlation between BTH dose and protection, in soil-drench application of BTH in rice seedlings against *Magnaporthe grisea.* The median effective dose (ED_{50}) for BTH was 183 mg a.i. L^{-1}. At the highest dose tested, 250 mg a.i. L^{-1}, no phytotoxic symptoms were observed on plants, however some authors have described phytotoxic effects. Wurms *et al*. (1999) and Ishii *et al*. (1999) demonstrated that prolonged use of BTH resulted in phytotoxic symptoms in cucumber, and the level of phytotoxicity seemed to be variable according to environmental conditions (light and temperature). Tally *et al*. (1999) indicated that under certain circumstances, loading rate (the total amount applied per season) may result in undesirable effects in some plants on the growth and development of a variety of crops (e.g. yield fruit reduction of green bell pepper, leaf bronzing on banana, stunting in burley type tobacco). However, reports of detrimental effects of benzothiadiazole on plant growth or yield are rare.

The efficacy of simultaneous (mixed) and consecutively (combined) sprays of plant defense activators (fosetyl-Al and benzothiadiazole), copper and antibiotics under controlled environment conditions depends on products, spray timing and dose. In mixed treatments the mixtures were sprayed at the best time interval for systemic compounds. The efficacy observed was the same for plant defense inducers applied alone or mixed with antibiotics and copper at half-reduced rates, indicating that the control of disease was mainly due to plant defense activators. BTH alone or mixed with antibiotics was the most effective treatment, and controlled the disease at the same level than streptomycin. In combined treatments each compound was sprayed at its optimal dose and timing. Systemic compounds were sprayed at the best time interval (7 days before inoculation) while antibiotics or copper were sprayed 2 hours before pathogen inoculation. This strategy optimized the effect of both compounds. The efficacy of plant defense activators was improved with antibiotics, since the same disease control level was obtained at half-reduced dose of

antibiotics. The effectiveness of fosetyl-Al and BTH was increased when combined with half-dose of kasugamycin, achieving the same control level as kasugamycin alone applied at higher dose (the double). The same effect was observed when plant defense inducers were combined with streptomycin. These results are in agreement with those from Thomson *et al*. (1998) who found that a combined strategy of BTH plus streptomycin provided 1.2 to 2 times better control than either BTH or streptomycin alone in orchard trials in apple, indicating that this would be logical since they each have a different mode of action. In the case of streptomycin the action is directed toward the pathogenic bacteria whereas BTH elicits the SAR response in the host plant. However, in our assays copper sulfate failed in disease control and when applied combined with fosetyl-Al or BTH was similarly effective than when applied alone, indicating a higher effect of systemic compounds than copper. Phytotoxicity is a serious problem on many plants when fosetyl-Al and copper products are used together or even when they are applied in separate sprays seven or fewer days apart. Because an interval shorter than 7 days may be necessary on some plants under certain environmental conditions, use of copper and acidic compounds on the same crop is not recommended.

The mixed strategy is more practical and easier to apply in orchard than the combined one. However, the best level of fire blight control was achieved with the combined strategy, and the effect of plant defense activators was improved with half-reduced rates of antibiotics.

Under greenhouse conditions only the combined strategy was tested, and was evaluated in pear and apple seedlings. In this experiment an experimental biocontrol agent was included and combined with BTH, and some changes in time spraying and dose of products were performed in order to optimize their efficacy in fire blight control. Plant defense inducers were sprayed two times (7 and 4 days before inoculation), the dose of BTH was increased to 150 mg a.i. L^{-1}, and the dose of kasugamycin in combined treatment was not half-reduced. The best results were obtained with BTH-kasugamycin combined spray, reducing the disease severity to similar levels than kasugamycin or BTH when applied alone, both in pear and in apple. Biocontrol agent combined with BTH also showed high effectiveness mainly in pear, probably due to the fact that it was selected in experiments carried out in pear, although the biocontrol agent alone showed the same efficacy on pear and apple. In this experiment we only tested the biocontrol agent as a product with local action because we sprayed it 2 hours before inoculation; in future assays it would be interesting to test its effectiveness increasing the time interval before inoculation and with multiple applications. Fosetyl-Al alone was the less effective product in pear and in apple.

In a field trial, combined sprays of benzothiadiazole with kasugamycin or with biocontrol agent did not produce any significant effect on disease control, and were the less effective treatments, while compounds applied alone (plant defense inducers, copper and kasugamycin) seemed to effectively reduce apple blossom blight. Thereby further experiments should be performed in order to confirm these preliminary results, and test new combinations, schedules and rates of compounds.

At the highest concentration tested for benzothiadiazole (100 mg a.i. L^{-1}) no inhibition of bacterial growth was obtained *in vitro*, although it was effective in disease control at doses of 75-150 mg a.i. L^{-1}. This confirms that the benzothiadiazole does not act directly against the pathogen. Moreover, to be effective, benzothiadiazole must be sprayed some days before infection indicating a systemic action. In our experiments phosphonate derivatives showed a weak *in vitro* activity against bacterial species, and two strains seems to be more sensitive than others, *X. arboricola* pv. *juglandis* and *P. syringae* pv*. phaseolicola* in comparison with results from Chase (1993) indicating the overall ranking of some plant pathogens in sensitivity to fosetyl-Al showing that *Xanthomonas* spp. are most sensitive, *Pseudomonas cichorii* is moderately sensitive, and *Erwinia* spp. are least sensitive. But in our results we did not find any relation by genus according to phosphonate sensitivity. At relatively high concentrations of phosphonates (0.32-0.63 mg HPO_3^{2-} L^{-1} or more) some inhibition of bacterial growth was observed *in vitro*. They are active in the plant at concentrations of 0.5-1.86 mg HPO_3^{2-} L^{-1}, which makes it unlikely that they are acting directly against the pathogen. One explanation may be that both fosetyl-Al and ethephon are broken down in the plant to a more toxic derivative, which then acts on the pathogen, although the balance of evidence indicates that it is not a powerful enough toxicant to explain the activity of fosetyl-Al and ethephon in the plant. On the other hand, there are strong indications that fosetyl-Al and ethephon are able to induce a resistant response in the plant (Bompeix *et al*., 1980; Guest, 1986; Dercks and Buchenauer, 1987; Balandin *et al*., 1995). Plants possess a number of natural defense mechanisms, and infection can only succeed if these defenses fail. Although fosetyl-Al controlled some diseases in some tests, results were erratic. Phytotoxicity was an additional problem when fosetyl-Al was applied to plants that were also treated with copper compounds either separately or together. Copper showed direct antibacterial effect *in vitro* against the bacteria tested. *Pseudomonas* spp. seems to be the most sensitive to antibiotics, *Xanthomonas* spp. showed moderate-low sensitivity and *Erwinia* spp. seems to have moderate sensitivity to streptomycin and kasugamycin.

Registration of additional compounds for fire blight control would be extremely important to growers, enabling them to alternate between bactericides and reduce the risk of development of resistant *E. amylovora* populations or phytotoxic effects of some conventional compounds. The improvement of application strategy of conventional and new compounds could also be an alternative.

CHAPTER 3

ULTRASTRUCTURAL AND MOLECULAR STUDIES OF PEAR RESPONSES TO *Erwinia amylovora* INFECTION AND TO PLANT DEFENSE ACTIVATORS

1. INTRODUCTION

The use of induced resistance in plants is a promising environment-friendly strategy for controlling plant diseases, including those caused by bacteria. Induced resistance, which is expressed systemically and/or locally, is biologically activated in response to necrotizing pathogens or root-colonizing soil bacteria (Hammerschimdt *et al.,* 2001). The best characterized systemic resistance is systemic acquired resistance (SAR). SAR can be activated by necrotic lesions, whether these are formed as part of the hypersensitive response (HR) in an incompatible interaction or as a symptom of disease in a compatible interaction (Ryals *et al.,* 1996). SAR can also be elicited by exogenous treatments with chemical inducers, such as salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA, CGA 41396), or benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH or acibenzolar-S-methyl; ASM, CGA 245704) (Ward *et al.*, 1991; Uknes *et al.*, 1992; Vernooij *et al.*,1995; Friedrich *et al.*, 1996; Lawton *et al.*, 1996). SAR is a mechanism in plants that, together with other defense mechanisms, provides broad-spectrum and long-lasting disease control (Table 3.1).

Table 3.1. Some biological characteristics of systemic acquired resistance (Lucas, 1999)

1. Induced by agents or pathogens causing necrosis e.g. local lesions
2. Delay of several days between induction and full expression
3. Protection conferred on tissues not exposed to inducer inoculation
4. Expressed as reduction in lesion number, size, spore production, pathogen multiplication, etc.
5. Protection is long-lasting, often for weeks or even months
6. Protection is non specific e.g. effective against pathogens unrelated to inducing agent
7. The signal for SAR is translocated and graft-transmissible
8. Protection not passed on to seed progeny; transmission to vegetatively propagated tissues has not been fully resolved

Cytological and molecular investigations provide valuable information on the mode of action of SAR inducer chemicals. Previous results shown in chapter 2, indicate the efficacy of benzothiadiazole and phosphonates in controlling fire blight and the possibility of their use as SAR inducers in rosaceous plants. So, in this chapter we study the ultrastructural changes and pathogenesis-related protein expression in pear tissues, after plant defense activator application (phosphonate derivatives and benzothiadiazole).

1.1. Migration and cytological changes due to infection by *Erwinia amylovora*

Fire blight is a vascular wilt disease. Pathogen invasion occurs primarily via natural openings in flowers (nectaries) or through wounds on young aerial vegetative parts. Numerous studies have been done to elucidate migration of *E. amylovora* in plant tissues. First it was assumed that migration of *E. amylovora* occurred in the phloem (Lewis and Goodman, 1965), although later reports confined bacterial movement exclusively to the xylem after artificial inoculation involving damage of the main vein (Goodman and White, 1981; Suhayda and Goodman, 1981a; Bogs *et al.,* 1998). Bogs *et al.* (1998) located bacteria in the xylem and observed bacterial aggregation causing a disruption of the vessel walls, presumably at the pit membranes, which are sensitive to changes in the vessel pressure. Probably, the migration of *E. amylovora* in host tissue changes according to the mode of penetration and the time after infection. After natural blossom infections, bacterial movement occurs mainly in the intercellular spaces, but they may also migrate into the xylem after wound infection (Vanneste, 1995). It is possible that the primary routes of infection are the xylem vessels and the later routes are other tissues. The main cytological changes due to *E. amylovora* infection are the initial appearance of fibril material in the xylem vessels, xylem occlusion later and xylem parenchyma plasmolysis finally (Goodman and White, 1981; Suhayda and Goodman, 1981b). Moreover, in the xylem vessels the bacteria are minimally exposed to the plant defense mechanisms because of their rapid migration and the lignified walls of the xylem cells. Besides, outbreaks of bacteria into the parenchyma could provide a mechanism of intensive colonization and destruction of the plant tissue, thereby enhancing the formation of fire blight symptoms (Bogs *et al.,* 1998). When established, bacteria multiply and progress in the intercellular spaces between parenchyma cells, leading to the rapid development of necrosis of infected tissues (Thomson, 1992). In susceptible cultivars, multiplication and progression of bacteria in parenchyma is followed by necrosis of progressively invaded tissues, whereas in resistant cultivars, the necrosis is restricted to the infection site.

1.2. Induction of resistance response with benzothiadiazole

The agrochemical industry has a long-standing interest in discovering compounds which might act indirectly against pathogens via endogenous plant defense pathways. Indeed, some compounds initially described as fungicides are now known to enhance defense responses rather than having a direct effect on the pathogen (probenazole). Biochemical and molecular elucidation of SAR has

provided the opportunity for a more targeted approach to the discovery of plant defense activators. The past few years have witnessed the discovery of a number of synthetic chemicals that act as strong elicitors of plant defense reactions, while being devoid of antimicrobial activity (Métraux *et al.*, 1991; Cohen *et al.*, 1994). With novel screening techniques for searching compounds, the benzo(1,2,3)thiadiazole-7-carboxylic acid derivatives have been identified as a new class of chemicals which stimulate the plant's own defense mechanism. Benzothiadiazole is the first synthetic chemical plant activator, developed for this novel disease control concept. Benzothiadiazole was identified by screening compounds that would protect plants from pathogen infections without having direct antifungal activity (Kunz *et al.,* 1997).

1.3. Pathogenesis-related proteins

Plant disease resistance against phytopathogenic organisms generally is associated with the induction of *de novo* defense mechanisms (Hutcheson, 1998). This induction presupposes recognition of the invading pathogen by receptors of the plant cell and the existence of signal-transduction pathways leading to the activation of plant defense genes. Initially, within minutes, pathogen recognition by the host triggers a variety of early defense responses, such as modification of the ion fluxes across the plasma membrane, lipid peroxidation, activation of antioxidant enzymes, protein phosphorilations, and production of reactive oxygen species in cells immediately confronted with the pathogen, leading to their death (the hypersensitive response; HR) (Grant and Mansfield, 1999; Nürnberger and Scheel, 2001). Within hours, these events are followed by the induction of a broad spectrum of defense reactions that confer local resistance against microbes (the localized acquired resistance; LAR) and involve adjacent cells surrounding the infection site. These include a cell wall reinforcement, the production of signaling secondary metabolites from the phenylpropanoid and octadecanoid pathways, such as salicylic acid and jasmonates, respectively, and the accumulation of components with antimicrobial activity such as phytoalexins and pathogenesis-related proteins (PR proteins) (Kombrink and Somssich, 1995). PR proteins can also be synthesized throughout the plant and lead to systemic acquired resistance, which represents the third line of defense. The accumulation of PR proteins is one of the best-characterized plant defense responses. PR proteins are involved in plant responses to biotic and abiotic stresses and are grouped into 17 families. Among them, we can find hydrolytic enzymes, chitinases and glucanases, as well as antimicrobial proteins of the thaumatin group, and the thionins (Table 3.2). Although expression patterns of PR genes vary among different plant species (Ryals *et al.*, 1996), the induction of genes PR-1, PR-2, and PR-5 by pathogens and chemicals occurs in most dicotyledons, and consequently these genes have often been used as markers of SAR onset (Ward *et al.*, 1991; Uknes *et al.*, 1992; Friedrich *et al.*, 1996; Lawton *et al.*, 1996). In monocotyledons, several homologues of the dicotyledonous PR genes have been identified. In particular, PR-1 and PR-5 homologues have been characterized in maize and barley, and found to be induced in both incompatible and compatible interactions (Casacuberta *et al.*, 1991; Hahn *et al.*, 1993; Muradov *et al.*, 1993; Bryngelsson *et al.*, 1994; Stevens *et al.*, 1996; Morris *et al.*, 1998).

Table 3.2. Recognized families of pathogenesis-related proteins (updated from van Loon and van Strien, 1999)

Family	Type member	Properties	Reference
PR-1	Tobacco PR-1a	antifungal	Antoniw *et al.*, 1980
PR-2	Tobacco PR-2	β-1,3-glucanases	Antoniw *et al.*, 1980
PR-3	Tobacco P, Q	chitinase type I, II, IV, V, VI, VII	Van Loon, 1982
PR-4	Tobacco 'R'	chitinase type I, II	Van Loon, 1982
PR-5	Tobacco S	thaumatin-like protein (TLP)	Van Loon, 1982
PR-6	Tomato Inhibitor I	proteinase-inhibitor	Green and Ryan, 1972
PR-7	Tomato P69	endoproteinase	Vera and Conejero, 1988
PR-8	Cucumber chitinase	chitinase type III	Métraux *et al.*, 1988
PR-9	Tobacco 'lignin-forming peroxidase'	peroxidase	Lagrimini *et al.*, 1987
PR-10	Parsley 'PR1'	'ribonuclease-like'	Somssich *et al.*, 1986
PR-11	Tobacco 'class V' chitinase	chitinase type I	Melchers *et al.*, 1994
PR-12	Radish Rs-AFP3	defensin	Terras *et al.*, 1992
PR-13	Arabidopsis THI2.1	thionin	Epple *et al.*, 1995
PR-14	Barley LTP4	lipid-transfer protein (LTP)	García-Olmedo et al., 1995
PR-15	Barley OxOa (germin)	oxalate oxidase	Zhang *et al.*, 1995
PR-16	Barley OxOLP	'oxalate oxidase-like'	Wei *et al.*, 1998
PR-17	Tobacco PRp27	unknown	Okushima *et al.*, 2000

The sequences of cloned fragments of defense genes from apple cv. Evereste and MM106 which encode PR proteins (chitinase, beta-1,3-glucanase, and thaumatine-like protein 5) or enzymes of the phenylpropanoid pathway (phenylalanine ammonia-lyase) have been described. The nucleic acid sequences of these fragments are available in GenBank and the accession numbers are listed in Table 3.3. There is more than one sequence for the same protein. Defense genes are known to belong to multigenic families; therefore, different groups of sequenced genes probably correspond to different isoforms.

Table 3.3. Accession numbers of defense genes described in *Malus* sp. (Venisse *et al.,* 2002)

cDNA[a]	Accession numbers	Name	Size (bp)
CHT	AF494395	class II chitinase (CHT-1) mRNA	296
GLU	AF494404	beta-1,3-glucanase (GLU-1) mRNA	320
PAL	AF494403	phenylalanine ammonia-lyase (PAL) mRNA	301
PR-5	AF494393	thaumatine-like protein 5 (PR-5) mRNA	663

[a] CHT: chitinase; GLU: β-1,3-glucanase; PAL: L-phenylalanine ammonia-lyase; PR-5: pathogenesis related protein 5

Several studies were done to know which is the mode of action of benzothiadiazole (BTH), mainly in annual plants, such as *Arabidopsis* sp., tobacco and cucumber. Little is known about how it acts in perennial plants (Brisset *et al.*, 2000). Tables 3.4 and 3.5 summarize PR protein induction and other plant defense mechanisms activated by benzothiadiazole in annual and perennial plants, respectively.

Table 3.4. Annual plants in which it has been demonstrated that benzothiadiazole activates disease resistance

Plant	Pathogen controlled	Effect	Reference
Strawberry (*Fragaria ananassa*)		Increase the levels of protective and health-promoting compounds	Karjalainen *et al.,* 2002
	Virus		
Arabidopsis thaliana	Turnip crinckle virus	Induction of PR-1, PR-2, PR-5	Lawton *et al.,* 1996
	Bacteria		
A. thaliana	*Pseudomonas syringae* pv. *tomato*	Induction of PR-1, PR-2, PR-5	Lawton *et al.,* 1996
Bean (*Phaseolus vulgaris*)	*Xanthomonas campestris* pv. *phaseoli*	Induction of chitinase, β-1,3-glucanase, and peroxidase	Siegrist *et al.,* 1997
Tomato (*Lycopersicon esculentum*)	*Pseudomonas syringae* pv. *tomato*	Induction of P4 mRNA accumulation	Fidantsef *et al.,* 1999
Cotton *(Gossypium hirsutum*)	*X. campestris* pv. *malvacearum*	Induction of β-1,3-glucanase	Colson-Hanks and Deverall, 2000
Pea (*Pisum sativum*)	*Pseudomonas syringae* pv. *pisi*	Induction of chitinase and β-1,3-glucanase	Dann and Deverall, 2000
Pepper *(Capsicum annuum*)	*X. campestris* pv. *vesicatoria*	Induction of resistance	Buonaurio *et al.,* 2002
	Fungus		
A. thaliana	*Peronospora parasitica*	Induction of PR-1, PR-2, PR-5	Lawton *et al.,* 1996
Tobacco (*Nicotiana tabacum*)	*Peronospora tabacina* *Phytophthora parasitica*	Induction of PR-1	Friedrich *et al.,* 1996
Bean	*Uromyces appendiculatus* *Rhizoctonia solani* *Colletotrichum lindemuthianum*	Induction of chitinase, β-1,3-glucanase, and peroxidase	Siegrist *et al.,* 1997
Tomato	*Fusarium oxysporum* f. sp. *radicis-lycopersici*	Formation of protective layers at sites of potential fungal entry	Benhamou and Bélanger, 1998b
Cucumber (*Cucumis sativus*)	*Pythium ultimum*	Sensitize plants to respond more rapidly and efficiently to pathogen attack	Benhamou and Bélanger, 1998a
Cucumber	*Colletotrichum lagenarium* *Cladosporium cucumerinum*	Increase of peroxidase activity	Ishii *et al.*, 1999
Melon (*Cucumis melo*)	Alternaria Fusarium Rhizopus	Decreased post harvest disease in melon by activating systemic resistance	Huang *et al.,* 2000
Strawberry	*Botrytis cinerea*	Decreased post harvest grey mold	Terry and Joyce, 2000
Pea	*Mycosphaerella pinodes* *Uromyces fabae*	Induction of chitinase and β-1,3-glucanase	Dann and Deverall, 2000
Cauliflower (*Brassica oleracea* var. *botrytis*)	*Peronospora parasitica*	Induction of PR-1, PR-2, PR-5	Ziadi *et al.,* 2001
Wheat (*Triticum aestivum*)	*Blumeria graminis* f. sp. *tritici*	Induction of WCI-1 to WCI-5	Görlach *et al.,* 1996
Wheat	*B. graminis* f. sp. *tritici*	Induction of phenylalanine ammonia-lyase (PAL) and peroxidase activity	Stadnik and Buchenauer, 2000
Maize (*Zea mays*)	*Peronosclerospora sorghi*	Activates expression of PR-1 and PR-5	Morris *et al.,* 1998
Rice (*Oryzae sativa*)	*Magnaporthe grisea*	Induction of *RCI1*, INA-induced gene	Schweizer *et al.,* 1999
	Root parasitic weed		
Sunflower (*Helianthus annuus*)	*Orobanche cumana*	Increased levels of chitinase class III	Sauerborn *et al.,* 2002

Table 3.5. Perennial plants in which it has been demonstrated that benzothiadiazole activates disease resistance

Plant	Pathogen controlled	Effect	Reference
Apple (*Malus domestica* var. Golden Delicious)		Induction of two subclasses of PR-10 transcripts	Ziadi *et al.*, 2001
	Bacterial		
Apple (*Malus domestica* var. Golden Delicious)	*Erwinia amylovora*	Activation of defense mechanism by induction of peroxidases and β-1,3-glucanases	Brisset *et al.*, 2000
	Fungus		
Japanese pear (*Pyrus pyrifolia* var. Nakai)	*Gymnosporangium asiaticum*	Increase of peroxidase activity	Ishii *et al.*, 1999
Durian trees (*Durio zibethinus*)	*Phytophthora palmivora*	Induce resistance	Arcade, 1999
Papaya (*Carica papaya*)	*Phytophthora palmivora*	Increase enzyme activity of chitinases and β-1,3-glucanases	Zhu *et al.*, 2002
Cashew (*Anacardium occidentale*)	*Colletotrichum gloeosporioides*	Induction of resistance	Lopez and Lucas, 2002

Further research will be necessary in order to understand how benzothiadiazole triggers the induction of resistance in pear. The experiments carried out in chapter 2 suggest that chemical plant defense inducers could be valuable in fire blight control. The aim of this chapter is to elucidate the mechanisms involved in their mode of action by means of histological, ultrastructural and molecular comparisons between compatible *Erwinia amylovora*-pear interaction and chemical-induced resistance responses on pear leaves. As phosphonate derivatives are also reported as plant defense inducers (Bompeix *et al*., 1980; Guest, 1986; Balandin *et al.,* 1995) their activity in *E. amylovora*-pear interaction at cytological and molecular level was also studied.

OBJECTIVES

As it has been pointed out, the main objective of this chapter is to determine the effect of benzothiadiazole and phosphonates in *Erwinia amylovora*-pear interaction at histological, ultrastructural and molecular level. In particular, structural changes in pear leaf tissues produced by *E. amylovora* infection, and ultrastructural modifications of pear tissues after product application will be studied and compared with the effects of copper and antibiotics. In addition, molecular studies on pear defense genes induced by benzothiadiazole have been included in an attempt to elucidate the action mechanism of benzothiadiazole.

2.1. ULTRASTRUCTURAL CHANGES IN PEAR LEAF TISSUES AFTER *E. AMYLOVORA* INFECTION

Histological and ultrastructural studies of *E. amylovora* infection in pear leaf tissues will permit us to differentiate changes in pear leaf tissues due to pathogen infection from those caused by chemicals (benzothiadiazole or others). Histological studies were carried out under light and electron microscopy (transmission and scanning). Leaf tissues were analyzed at different time intervals after *E. amylovora* inoculation. Sample processing for electron microscope observation was carried out by the Technical Research Services of the Universitat de Girona.

2.1.1. Plant material

Leaves from young shoots of 2 year old pear (*Pyrus communis*) plants cv. Conference, clone CAV were used. Plants were forced to shoot 15 days to 1 month before inoculation, in order to obtain young shoots. These plants had the same characteristics as those used in previous tests (Chapter 2).

2.1.2. Bacterial inoculum and plant inoculation

The virulent strain EPS101 of *E. amylovora* was used. Bacteria were cultured at 25 ºC for 24 h on solid Luria-Bertani medium (Maniatis *et al*., 1982). Bacterial inoculum was prepared in sterile distilled water to yield a concentration of 10^8 cfu mL^{-1}. Inoculation was performed by cutting plant leaves with scissors soaked in the bacterial suspension (as described in p. 61). Leaves of control plants were cut with scissors soaked in sterile water.

2.1.3. Histological analysis

To observe the ultrastructural changes during *E. amylovora* infection fragments of leaf (0.5 cm long x 0.1 cm wide, including the midrib) were taken 0, 2 and 4 days after bacterial inoculation (Figure 3.1). Six samples were collected each time.

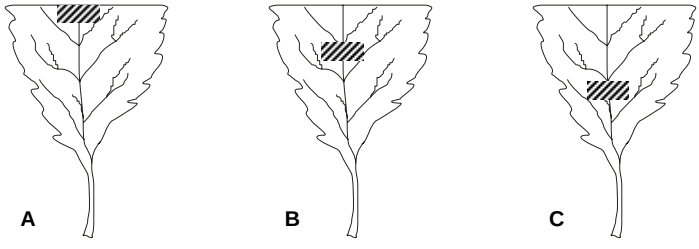


Figure 3.1. Small fragments of the leaf were collected beyond the necrosis site (hatched rectangle) and processed for light and electron (transmission and scanning) microscopy. Samples were collected; 0 (**A**), 2 (**B**) and 4 (**C**) days after bacteria inoculation.

Light and transmission electron microscopy. Plant material was fixed in 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.1-7.4) for 4 h at 4ºC. Post fixation was done in 1% osmium tetroxide in 0.1 M cacodylate buffer, and dehydration in a graded acetone series. For light microscope observations, samples were embedded in Spurr resin. Resin blocks were cut in semithin sections (1-2 μm) with a glass knife ultramicrotome (OMU-2, Reichert, Austria). These sections were stained with 0.5% methylene blue for a few seconds, and then examined with a light microscope (DMR-XA, Leica, Germany) to locate the interesting part of tissue.
For electron microscopy, ultrathin sections (60-90 nm) from resin blocks were cut with a Diatome diamond knife ultramicrotome (OMU-2, Reichert, Austria). The sections were collected on 200 mesh copper grids. The grids were placed in 2% uranyl acetate for 30 min, followed by Reynold's lead citrate for 10 min. The stained sections were examined with a transmission electron microscope (TEM) operating at 60 kV (EM 910, Zeiss, Germany). Image capture was performed by photo negatives on the TEM using an electron microscope film (4489, Eastman Kodak Co., NY, USA) 8.3 x 10.2 cm sheet.

Scanning electron microscopy. Pear leaf samples were fixed in 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.1-7.4) for 4 h at 4ºC, and dehydrated in a graded ethanol series. Samples were critical point dried with CO_2 in a pressure bomb (K850, Emitech, England). The specimens were mounted on metal stubs with colloidal silver (Electrodag 1415, Acheson, Holland) followed by gold sputter coat depending on thickness (ca. 30 nm) in a sputter system (K550, Emitech, England). The observations were done with a scanning electronic microscope (SEM) with 3.5 mm of resolution and at 15 kV of acceleration voltage (DSM 960A, Zeiss, Germany). Image capture and measurements were accomplished using a digital image acquisition system by Quartz PCI program (Version 5.1, Quartz Imaging Co., Canada).

2.2. Ultrastructural changes in pear leaf tissues after application of chemical inducers of plant defense response

Cytological events developed in pear leaf tissues after plant defense inducer application were investigated, by scanning and transmission electron microscopy observations. Ultrastructural studies were performed in leaf tissues of pear plants chemical sprayed and infected with virulent strain of *E. amylovora*. Samples of chemical sprayed and inoculated leaves were taken two and four days after inoculation. Benzothiadiazole and phosphonate derivatives were compared with other chemicals previously tested for fire blight control such as copper derivatives and streptomycin.

2.2.1. Plant material and bacterial inoculation

Leaves from vigorous shoots of 2 year-old pear plants cv. Conference were sprayed with chemicals, and inoculated with *E. amylovora* EPS101, as previously described (p. 61). Leaf samples were taken at different times after bacteria inoculation and processed for light and electron microscopy.

2.2.2. Products

Pear plants were uniformly sprayed to runoff with aqueous solutions of chemicals with a 1 L hand sprayer. Products tested were fosetyl-Al 1.86 g HPO_3^{2-} L^{-1}, ethephon 0.5 g HPO_3^{2-} L^{-1}, benzothiadiazole 100 mg a.i. L^{-1}, copper sulfate 1.5 g Cu L^{-1} and streptomycin 100 mg a.i. L^{-1}. All systemic compounds (fosetyl-Al, ethephon and BTH) were sprayed, 7 and 2 days before bacteria inoculation, and the others were applied 2 hours before bacteria inoculation. Sampling times were 2 and 4 days post-inoculation, for all treatment except for phosphonates, in which samples were only obtained 4 days after inoculation. Product-sprayed and non-pathogen-inoculated plants were used as controls.

2.2.3. Microscopic observations

Six samples of pear leaves per treatment were taken and processed for light and, scanning and transmission electron microscopy as previously described (p. 96).

2.3. MOLECULAR STUDIES OF PEAR LEAVES IN RESPONSE TO BENZOTHIADIAZOLE AND ETHEHPON APPLICATION

Expression of genes coding for pathogenesis related protein (PR genes) was used as a hallmark indicator of systemic acquired resistance induction in response to benzothiadiazole (BTH) and ethephon application. Since no marker genes of BTH and ethephon-induced resistance in pear are presently available, we decided to study the expression pattern of the same PR genes described by Venisse *et al*. (2002) in apple.

2.3.1. Plant material

Two-year-old cv. Conference (CAV clone) micropropagated pear plants (Agromillora Catalana, SA, Barcelona, Spain) were grown in a greenhouse under controlled environment conditions.

2.3.2. Chemical sprays

Bottom leaves of plants were sprayed with aqueous solutions of benzothiadiazole 200 mg a.i. L^{-1} and ethephon 0.5 g HPO_3^{2-} L^{-1}. One and five days after product application leaf samples were obtained and processed. Water-sprayed plants were used as control.

2.3.3. Determination of resistance induction

25-50 mg of pear leaf tissue was taken from the top leaves (non-sprayed) of chemical-treated pear plants (Figure 3.2). Samples were processed for chitinase (CHT), β-1,3-glucanase (GLU), L-phenylalanine ammonia-lyase (PAL), and thaumatin-like protein (PR-5) gene expression.

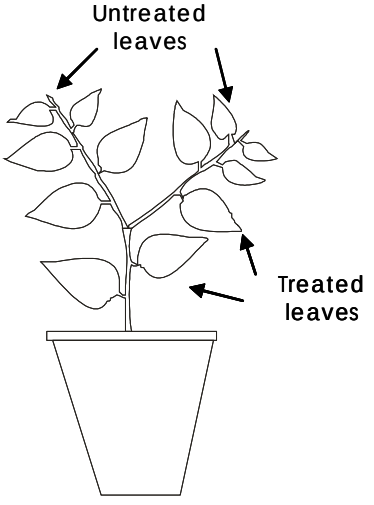


Figure 3.2. Scheme of the different leaves considered in the study. Chemical sprayed (treated leaves) and sampled leaves (untreated leaves) of pear plants processed for molecular studies on resistance induction after chemical application.

2.3.4. RNA extraction

Total RNA was extracted from 25-50 mg pear leaf tissue using the Plant RNeasy® kit (Qiagen, Hilden, Germany). Samples were ground to a fine powder in liquid nitrogen, and processed according to the manufacturer's instructions. An optional step of DNase (RNase-free) treatment was also performed. The extracted RNA was dissolved in 40 μL of water (DEPC treated), and stored at -80 °C for further analysis. Total RNA integrity; concentration and purity of each sample was determined by agarose gel electrophoresis (1.2% wt/vol); and spectrophotometrically by measuring the absorbance ratio A260/A280, respectively.

2.3.5. Reverse transcription-polymerase chain reaction analysis

First, total RNA was treated in the presence of oligod$(T)_{16}$ 2.5 μM at 75 ºC for 5 min. Further, the volume was increased to 60 μL containing 2.5 mM of $MgCl_2$, 0.5x buffer, 1 mM of each dNTPs, 60 U of RNase inhibitor and 150 U of MuLV RTase (Applied Biosystems, CA, USA). The mixture was incubated at 42 ºC for 15 min, followed by 5 min at 99 ºC and 5 min at 5 ºC. PCR amplifications were carried out using appropriate primers (Table 3.6). 10 μL of cDNA was used in a final volume of 50 μL of PCR mix containing 1x PCR buffer, 0.2 mM of each dNTPs, 1 μM of each primer (forward and reverse) and 2.5 U of Taq polymerase (AmpliTaq®, Applied Biosystems). PCR cycling conditions consisted in an initial denaturation step of 5 min at 95 ºC followed by 35 cycles of 30 s

at 95 ºC, 1 min at Tm ºC (Table 3.6), 1 min at 72 ºC, and a final elongation step of 15 min at 72 ºC. The amplification was done on a thermocycler (Gene Amp® PCR System 9700, PE, Applied Biosystems, CA, USA). The primer sequences were based on the specific *Malus* sp. sequence of Venisse *et al*. (2002).

Table 3.6. Primer sequences from *Malus* sp. used for polymerase chain reaction (Venisse *et al.,* 2002; Rosati *et al.,* 1997)

cDNA[a]	Specific *Malus* sp. primer sequence[b]	Tm (ºC)	Size (bp)
CHT	for 5'- GGT CAA ACT TCT CAT GAA AC -3' rev 5'- GGG GTC ATC CAG AAC CA -3'	50	296
GLU	for 5'- TAT GCT CTT TTC ACA GCT CCG -3' rev 5'- CAA TGT TTC TCA AGC TCT GG -3'	50	320
PAL	for 5'- GAG GCA TTT GGA GGA GAA CTT G -3' rev 5'- TCA ACC TCT TAA GGC AAA AGC GC -3'	50	301
PR-5	for 5'- AAC ACT GTT TGG CCA GGA ACC -3' rev 5'- GTT GAA GCC GTC AAC AAG GG -3'	55	309
EF 1α	for 5'- ATT GTG GTC ATT GGY CAY GT -3' rev 5'- CCA ATC TTG TAV ACA TCC TG -3'	58	700

[a] CHT: chitinase; GLU: β-1,3-glucanase; PAL: L-phenylalanine ammonia-lyase; PR-5: pathogenesis related protein 5; EF 1α: elongation factor 1 α

[b] Degenerate nucleotides: Y = C or T; V = G or T; R = A or G

Eight μL of the PCR product were analyzed by gel electrophoresis and stained with ethydium bromide (1 μg mL^{-1}) in 1x TAE. Agarose gels were visualized with a UV transilluminator, and gel images were captured using the Image Capture System (DC120, Eastman Kodak Co., NY, USA) and processed with 1D Image Analysis System (Eastman Kodak Co., NY, USA).

2.3.6. PR gene expression

Expression of target genes was studied by reverse transcription-polymerase chain reaction (RT-PCR) as described above. A constitutive gene (i.e. elongation factor 1 alpha gene: *Ef 1α*) was analyzed in parallel as control it was amplified using a degenerate primer pair (Table 3.6). The genes analyzed coded for one of the enzyme from the phenylpropanoide pathway; phenylalanine ammonia-lyase (PAL) and three PR-proteins; chitinase (CHT), β-1,3-glucanase (GLU) and pathogenesis-related protein 5 (PR-5). Global expression of each gene was analyzed by PCR using these specific *Malus* sp. primers and PCR cycling conditions described previously, with 25 cycles, after checking that exponential increase of PCR products was still recorded after 35 cycles. To check that PCR product was the defense gene it was sequenced. Sequencing of the PCR products was carried out using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA), and processed according to the manufacturer's instructions. The genetic analyzer (ABI PRISM® 310, Applied Biosystems, CA, USA) used belonged to the Molecular Biology Service of the Integrated Research Services of the Universitat the Girona.

3. RESULTS

3.1. ULTRASTRUCTURAL CHANGES IN PEAR LEAF TISSUES DUE TO *E. AMYLOVORA* INFECTION

Observations on the 2 mm segment from the bacteria inoculated leaf cut side revealed structural changes at 2 and 4 days after inoculation. Several changes were detected in the tissue structure of pear leaves with light and electron (scanning and transmission) microscope.

3.1.1. Light microscopy

Light microscope observations of cut but non-bacteria-inoculated pear leaves did not show changes in tissue structure (Figure 3.3.A). Two days after bacterial inoculation no change in the tissue structure was observed (Figure 3.3.B). Our observations indicated that the main changes in the pear leaf structure took place four days after *E. amylovora* inoculation. Spongy parenchyma and vein parenchyma were the most affected tissues, displaying a general cellular disorganization, while upper epidermis and palisade parenchyma maintained their structure partially complete. It seemed that spongy parenchyma was more susceptible than the palisade region (Figures 3.3.C). Lumen of the xylem vessel was not clear and showed some stained substance. Apparently central vein also showed some disorganization (Figure 3.3.D).

3.1.2. Electron microscopy

In the non-inoculated leaves no bacterial cells were observed (Figures 3.4.A and B). Transmission electron micrographs indicated no modification of cellular organization and all tissue structure was well organized. Xylem vessels did not show any fibrillar or electron-dense structure and the surface of the secondary thickening of the xylem was not altered. While two days after pathogen inoculation some bacterial cells included in an amorphous matrix were found in the vein cells, important changes in tissue structure were not observed (Figures 3.4.C and D). Important ultrastructural changes occurred four days after *E. amylovora* inoculation, consisting of a general disorganization of vein tissue (Figure 3.5.A). The bacteria rapidly proliferate, since bacterial cells were observed outside the xylem vessels, on the surface of parenchyma cells (Figure 3.5.B). A higher number of bacterial cells appeared on the surface of plant cells, of which, when observed in magnification, masses of bacteria formed aggregates and some bacteria were in the process of division (Figures 3.5.C and D).

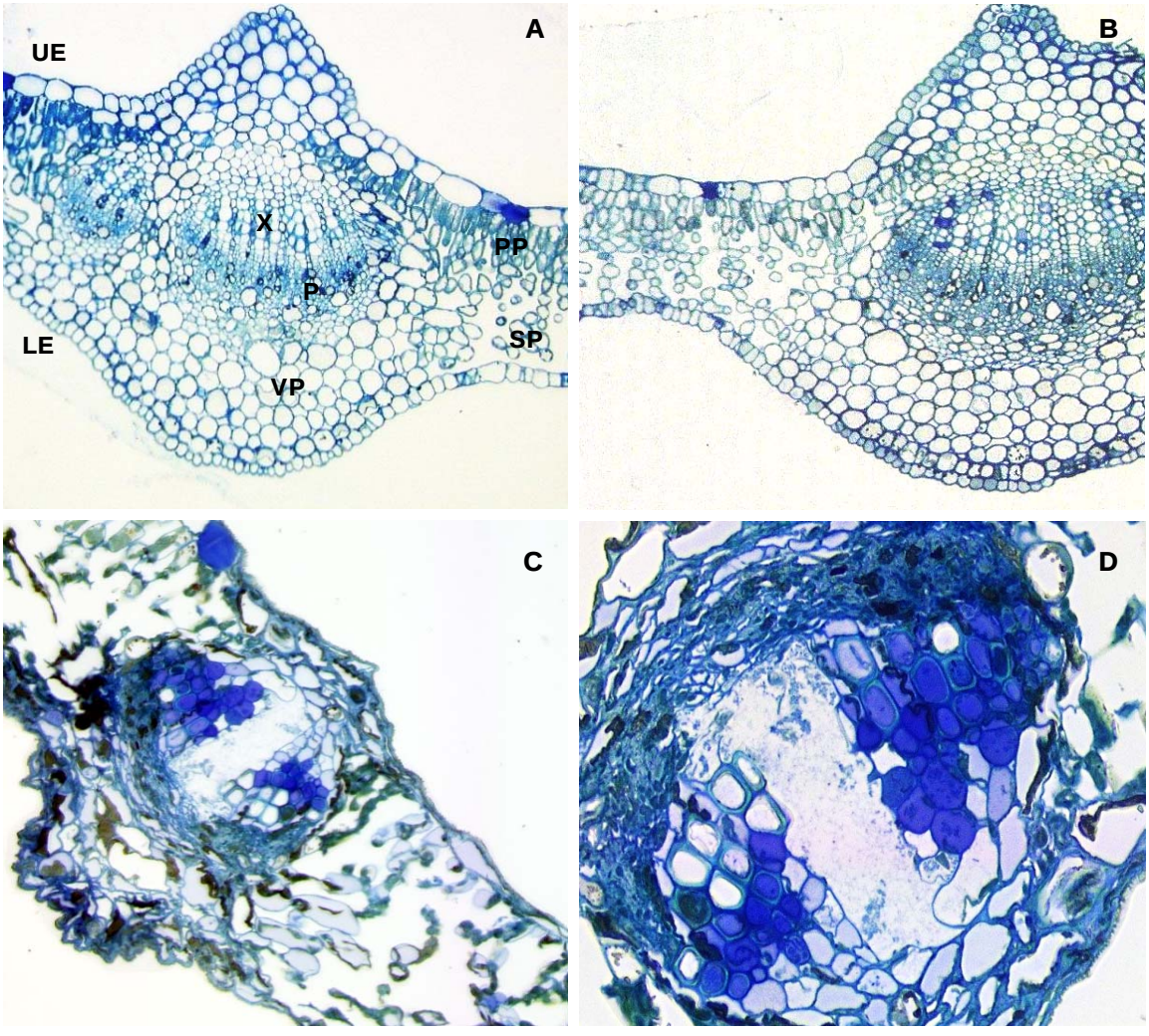


Figure 3.3. Methylene blue-stained light micrographs of cross sections of cv. Conference pear leaf at different time intervals after *E. amylovora* inoculation. **A:** Non-inoculated control, **B:** two days after pathogen inoculation, **C:** four days after pathogen inoculation showing tissue structure disorganization (x428), and **D:** higher magnification of the vein zone four days after pathogen inoculation (x214). LE: lower epidermis; UE: upper epidermis; PP: palisade parenchyma; SP: spongy parenchyma; VP: vein parenchyma; X: xylem; P: phloem.

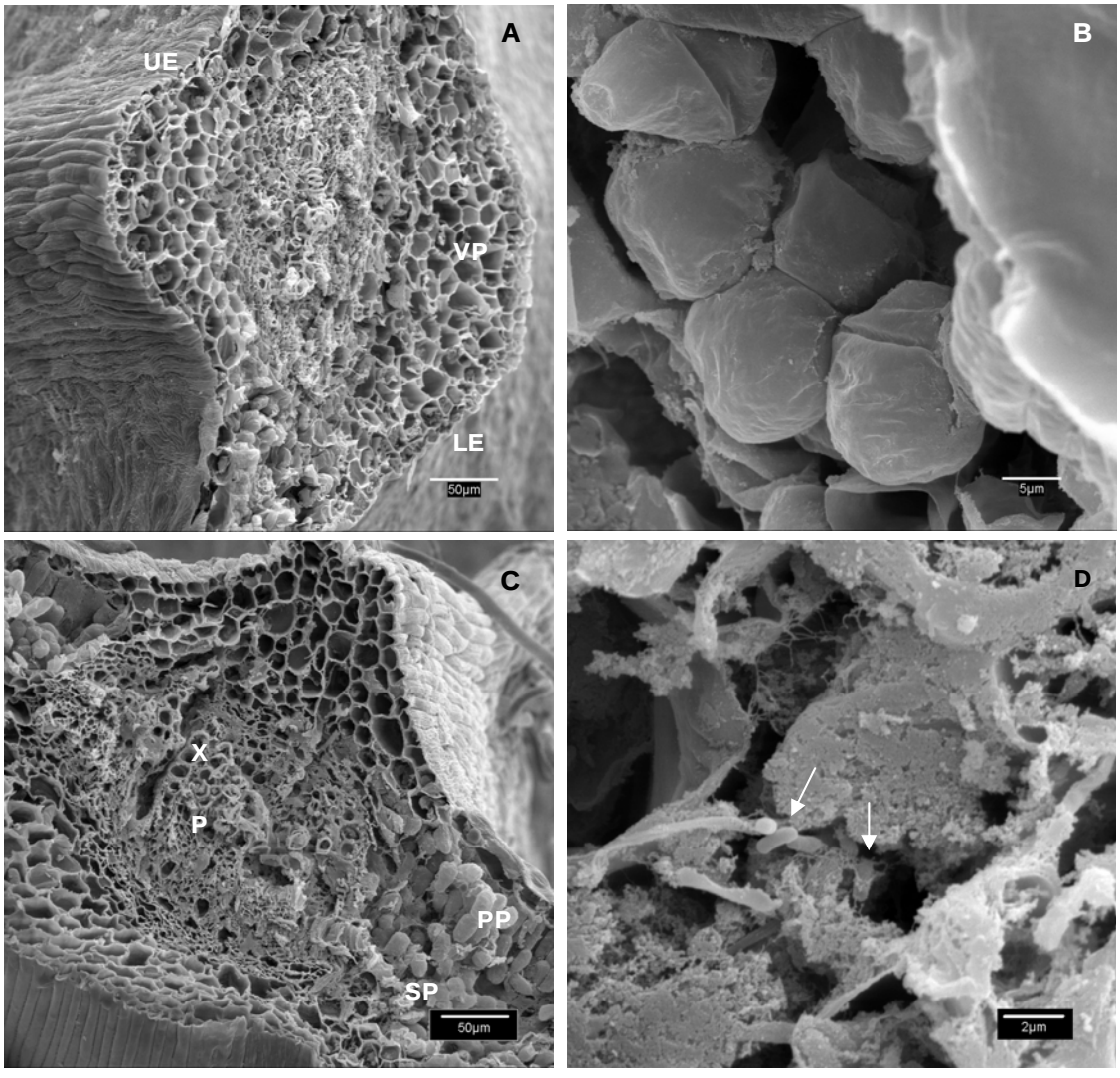


Figure 3.4. Scanning electron micrographs of pear leaf at different time interval after *E. amylovora* inoculation. **A** and **B:** Non-inoculated control, **C** and **D:** infected tissues two days after pathogen inoculation. White arrows: bacterial cells. LE: lower epidermis; UE: upper epidermis; PP: palisade parenchyma; SP: spongy parenchyma; VP: vein parenchyma; X: xylem; P: phloem.

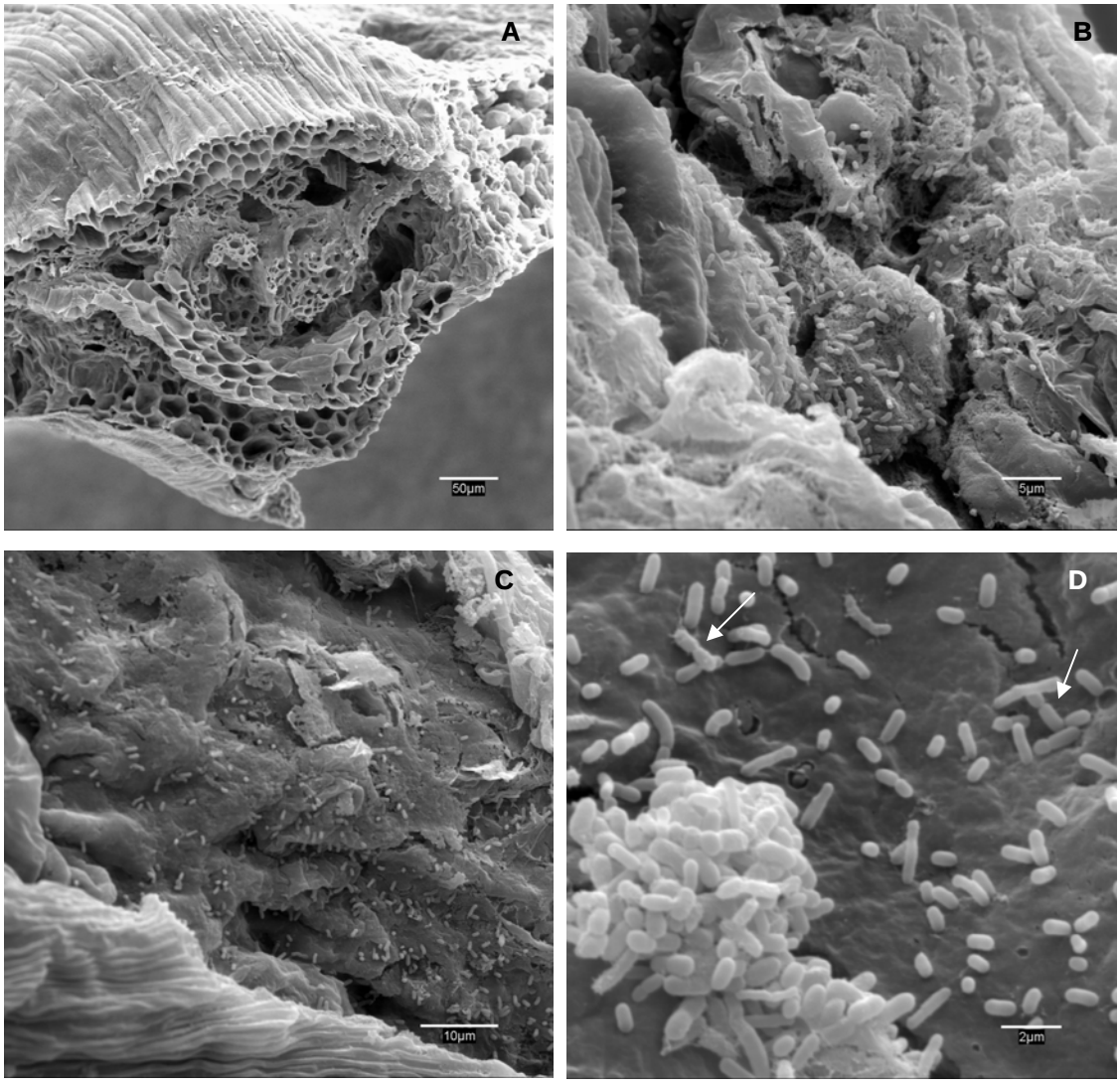


Figure 3.5. Scanning electron micrographs of *E. amylovora*-infected pear leaf four days after inoculation. **A:** General view of vein zone, **B** and **C:** high amount of bacterial cells on the plant cells surface, and **D:** bacteria aggregates. White arrows: some bacterial cells in division.

Transmission electron micrographs (Figures 3.6, 3.7, 3.8 and 3.9) revealed several changes in xylem vessels and in xylem parenchyma cells, two or four days after pathogen inoculation. In non-inoculated samples the surface of secondary wall of xylem vessels was smooth (Figures 3.6.A and B). Two days after inoculation further structural changes were observed, and some bacterial cells were present in intercellular spaces. The ultrastructural modifications included the appearance of some granular ground substance in the lumen of xylem vessels containing bacteria, occasionally this vessel-occluding substance could be seen in xylem vessels of inoculated leaves even though no bacteria were visible, and the appearance of a brushlike array of fibrils extending from the surface of the secondary wall and the helical secondary wall thickenings (Figures 3.7.A and B). Four days after inoculation, an intensified cellular disruption was observed. The ultrastructural modifications detected included intense plasmolysis of xylem parenchyma cells that bordered xylem vessels with lumens containing bacteria. The brushlike fibrillar structure on the wall surfaces of xylem vessels became granular in nature. Part of them appeared to become distributed through and contribute to the increased density of the ground substance in the vessel lumen in which the bacteria were embedded. The surface of the secondary thickening wall of the xylem vessel was not smooth and showed rough appearance which seemed the brushlike fibrils that had become more granular (Figure 3.9.A and B). Rupture of the xylem vessels released bacteria into the intercellular space and the space developed from the collapse of xylem parenchyma (Figure 3.9.C and D). Plasmolysis was most frequently noted in those parenchyma cells that bordered xylem vessels containing bacteria. The plasmolyzed protoplasts of xylem parenchyma cells were even aggregated and distorted.

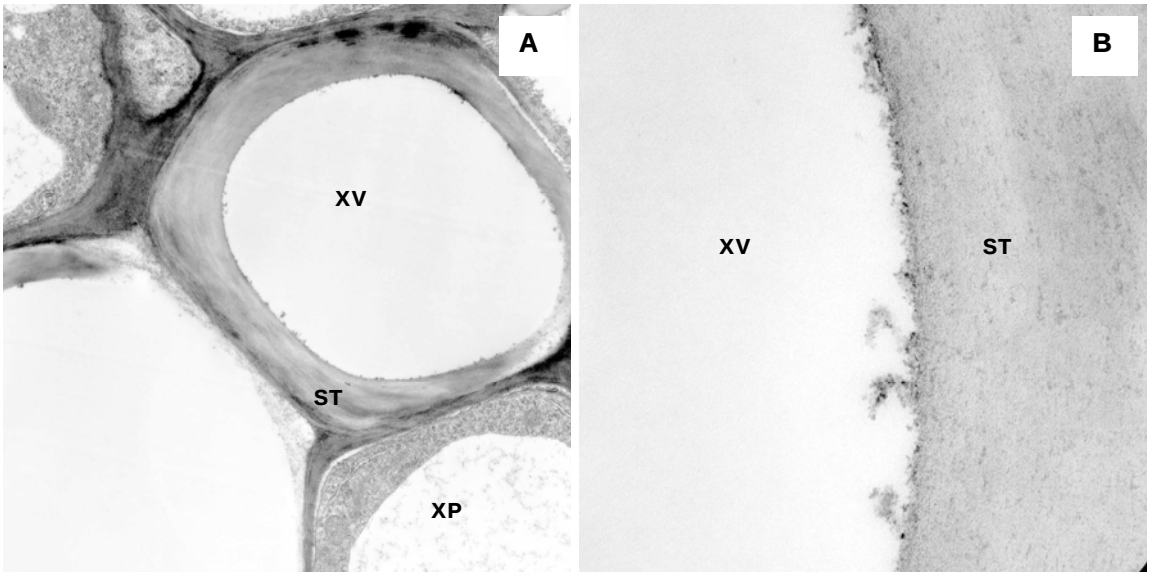


Figure 3.6. Transmission electron micrographs of non-*E. amylovora*-infected cv. Conference pear leaf. **A:** Xylem vessel (XV) lumens are entirely clear, the surface of the secondary thickenings (ST) of the XV is smooth, and surrounding healthy xylem parenchyma (XP) cells show no signs of plasmolysis (x8,000). **B:** Higher magnification showing smooth secondary thickening (x80,000).

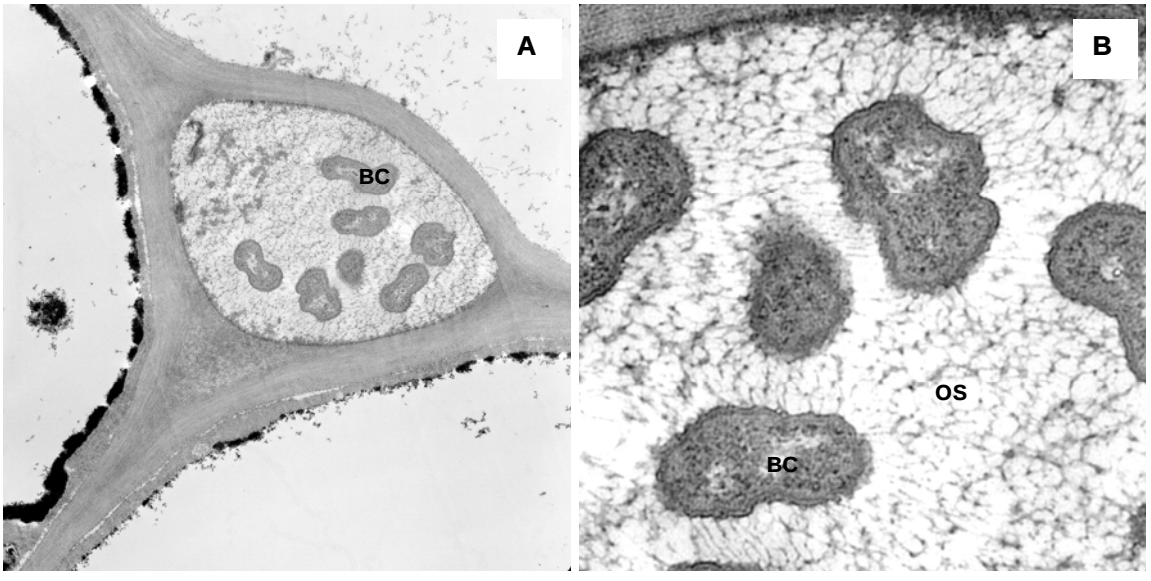


Figure 3.7. Transmission electron micrographs of *E. amylovora*-inoculated cv. Conference pear leaf two days after inoculation. Images correspond to tissue located 2 mm from the inoculation point. **A:** Xylem vessel lumens with several bacterial cells (BC) (x12,500). **B:** Higher magnification of bacterial cells, surrounded by occluding substance (OS) (x50,000).

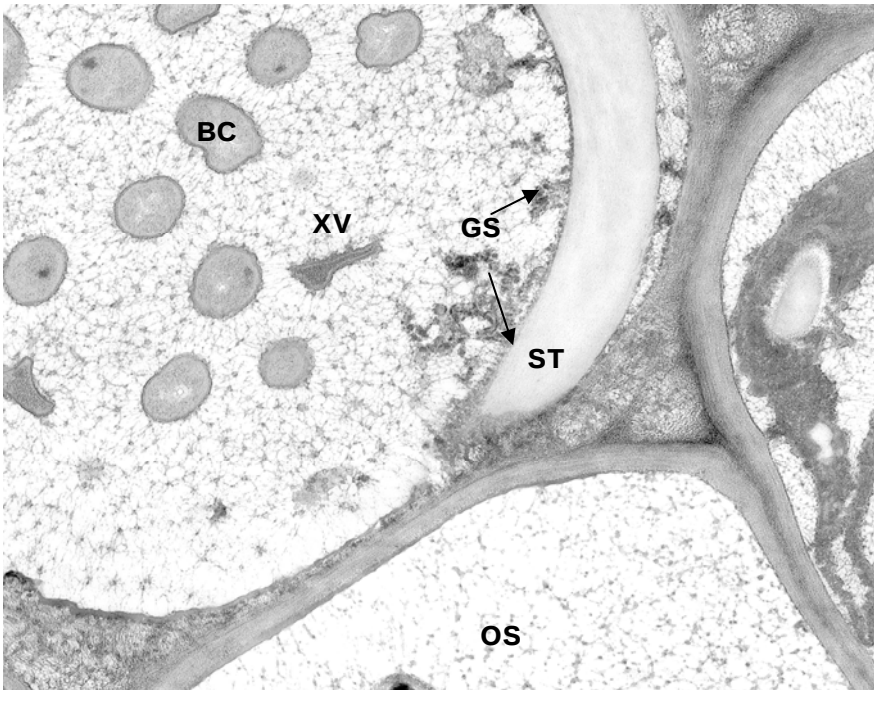


Figure 3.8. Transmission electron micrograph of *E. amylovora*-inoculated cv. Conference pear leaf four days after infection. Image corresponds to tissue located 2 mm from the inoculation site. **A:** The xylem vessel (XV) contains several bacterial cells (BC) and a great amount of occluding substance (OS) and granular substance (GS, arrows) on secondary thickening surface (ST) (x12,500).

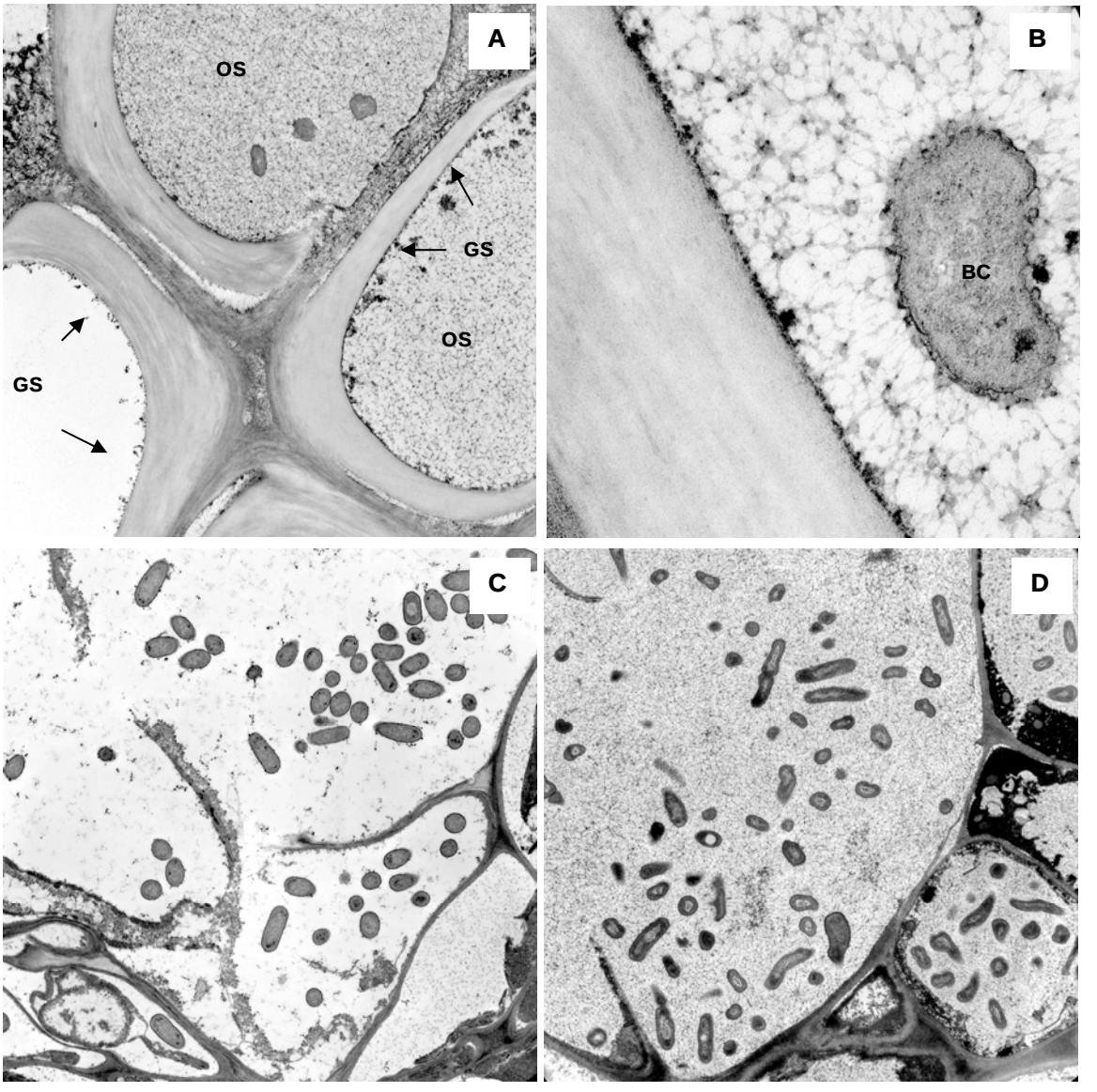


Figure 3.9. Transmission electron micrographs of *E. amylovora*-inoculated cv. Conference pear leaf four days after infection. **A:** Xylem vessel containing bacterial cells surrounded by heavier deposits of occluding substance (OS). The presence of granular substance (GS) is clear in these vessels (arrows) (x8,000). **B:** Higher magnification of the OS between bacterial cell (BC) and secondary thickening wall (x65,000). **C** and **D:** Middle lamella and secondary wall have ruptured permitting bacteria to spill into what will become enlarged the intercellular space (x5,000).

3.2. Ultrastructural changes in pear leaf tissues after application of chemical inducers of plant defense response

The effect of benzothiadiazole and phosphonates (fosetyl-Al and ethephon) on pear leaf tissues, compared to copper and streptomycin were studied at histological and ultrastructural level. Samples were taken after chemical treatment followed by *E. amylovora* inoculation. Non-bacterial-inoculated and chemical-sprayed plants were also included. Observations were made on the 2 mm segment from the bacteria inoculation point in a leaf.

Light and electron microscope observations indicated that applications of plant defense inducers (benzothiadiazole and phosphonate derivatives), copper sulfate and streptomycin did not produce any alteration or modification in pear leaf tissue when pathogen infection was not produced (non-*E. amylovora*-inoculated plants) (Figures 3.11.A, 3.12, 3.15.A, 3.18.C, 3.19.A and 3.20.A). Conversely, different types of alteration were observed in infected host cells depending on the product applied. As observations of leaf tissues were performed two and four days after *E. amylovora* inoculation, a more intense cell reaction was observed in later samples. Pear leaf changes included tissue and cell disruption due to bacteria colonization, and cellular, mainly cytoplasmic and cell wall, modifications, induced by the activity of some chemicals in *E. amylovora*-pear interaction.

Light microscopy showed that benzothiadiazole treatment did not produce any important structural change (Figure 3.10.A, B and C) and in comparison with the non-treated control, no differences were observed. Ultrastructural modifications in leaves from benzothiadiazole-sprayed plants were observed at two and four days after pathogen inoculation. An intense methylene blue-stained substances and small size particles were observed in xylem vessels (Figure 3.10.D) and some vein parenchyma cells had become disorganized and lost their structure, under light microscope. Scanning electron micrographs revealed that no important structural changes occurred in pear leaf cells after *E. amylovora* inoculation and that few bacterial cells were found under the surface of palisade parenchyma, whereas a higher amount of bacteria was located in xylem vessels (Figures 3.11.B, C and D). Electron microscopy confirmed the light microscope observations and revealed additional ultrastructural details. In the absence of pathogen challenge, treatment of plants with benzothiadiazole failed to induce visible cellular changes, as judged by the absence of typical wall appositions or intercellular space occlusions. After bacterial inoculation, some bacterial cells were found inside the xylem vessels or xylem parenchyma cells, although in lesser amounts than in non-benzothiadiazole-treated control. It is remarkable that, two days after pathogen inoculation, some bacterial cells located in xylem vessels were bordered by a clear zone without the typical occluding substance. This well defined clear zone around bacterial cells included thin fibrils surrounding bacterial cell wall (Figure 3.13.A and B). It was difficult to find bacterial cells in pear leaf tissue four days after bacterial inoculation, and some bacterial cells found in xylem parenchyma cells seemed to have lost their structure (Figure 3.13.D). Some xylem vessels contained in their lumen a matrix

of granular substance and heavy electron-dense particles. There was also observed a loss of xylem vessel cell wall structure, and the presence of a granular substance around the lumen of the vessel (Figure 3.13.C). These microscopic observations indicated that treatment of pear plants with benzothiadiazole reduced the rate and extent of *E. amylovora* colonization and triggered the elaboration of structural barriers. One of the most noticeable ultrastructural features of the benzothiadiazole-induced resistant reaction was the formation of electron-dense wall appositions. In conclusion, the benzothidiazole *per se* did not produce any important structural changes and in addition it delayed the structural disorganization caused by *E. amylovora* infection.

Changes in leaves treated with phosphonate derivatives were observed only four days after bacteria inoculation. After treatment with fosetyl-Al and pathogen inoculation, several changes were observed at cytoplasmic level of the upper epidermis and vein parenchyma cells. Light microscopy observations of these cells revealed that they were intense methylene blue-stained and that their cytoplasm was disorganized (Figures 3.14.C and D). Scanning electron micrographs of phosphonate treated plants (Figure 3.15) indicated the absence of bacterial cells, four days after pathogen inoculation, and the presence of an amorphous substance among parenchyma cells (Figure 3.15.D). Transmission electron micrographs of ethephon treated pear leaves showed some degree of cytoplasmic disorganization four days after pathogen inoculation and the presence of several bacterial cells inside the xylem vessels, parenchyma cells and in the intercellular space (Figure 3.16). However, pear plants sprayed with fosetyl-Al had a higher amount of matrix substance inside the xylem vessels containing bacterial cells, and the bacterial cell wall somewhat disorganized (Figure 3.17). It is remarkable that the matrix substance seemed more structured and dispersed than the occluding substance of non-treated control four days after inoculation. Additionally some electron-dense appositions were observed on the surface of secondary wall of xylem vessel (Figure 3.17.C) and inside parenchyma cells (Figure 3.17.D).

Finally, copper sprayed plants inoculated with *E. amylovora* did not present any structural modification, xylem vessels were clear and few bacteria were found in parenchyma cells, where the presence of an amorphous substance was detected, which was not previously seen in non-copper-treated plants (Figures 3.18.A and B). However, under transmission electron microscope a lower amount of occluding substance was observed in comparison with the non-treated control at the same time (Figure 3.20). Streptomycin-sprayed plants showed differences in leaf tissues compared to non-treated plants. Parenchyma cells lost their turgidity and the tonoplast seemed disorganized (Figure 3.18.C). Four days after pathogen inoculation some xylem vessels were occluded by a stained substance, but a general disorganization of tissue structure was not observed as occurred in non-treated-control plants (Figure 3.18.D). No bacterial cell was observed in parenchyma cells or intercellular space, while aggregates of non-defined substance were observed in the intercellular spaces (Figure 3.19.D) under scanning electron microscope. Transmission electron micrographs confirmed that, no changes in xylem tissue occurred after streptomycin application and *E. amylovora* inoculation since a well defined matrix was observed four days after pathogen inoculation (Figure 3.21).

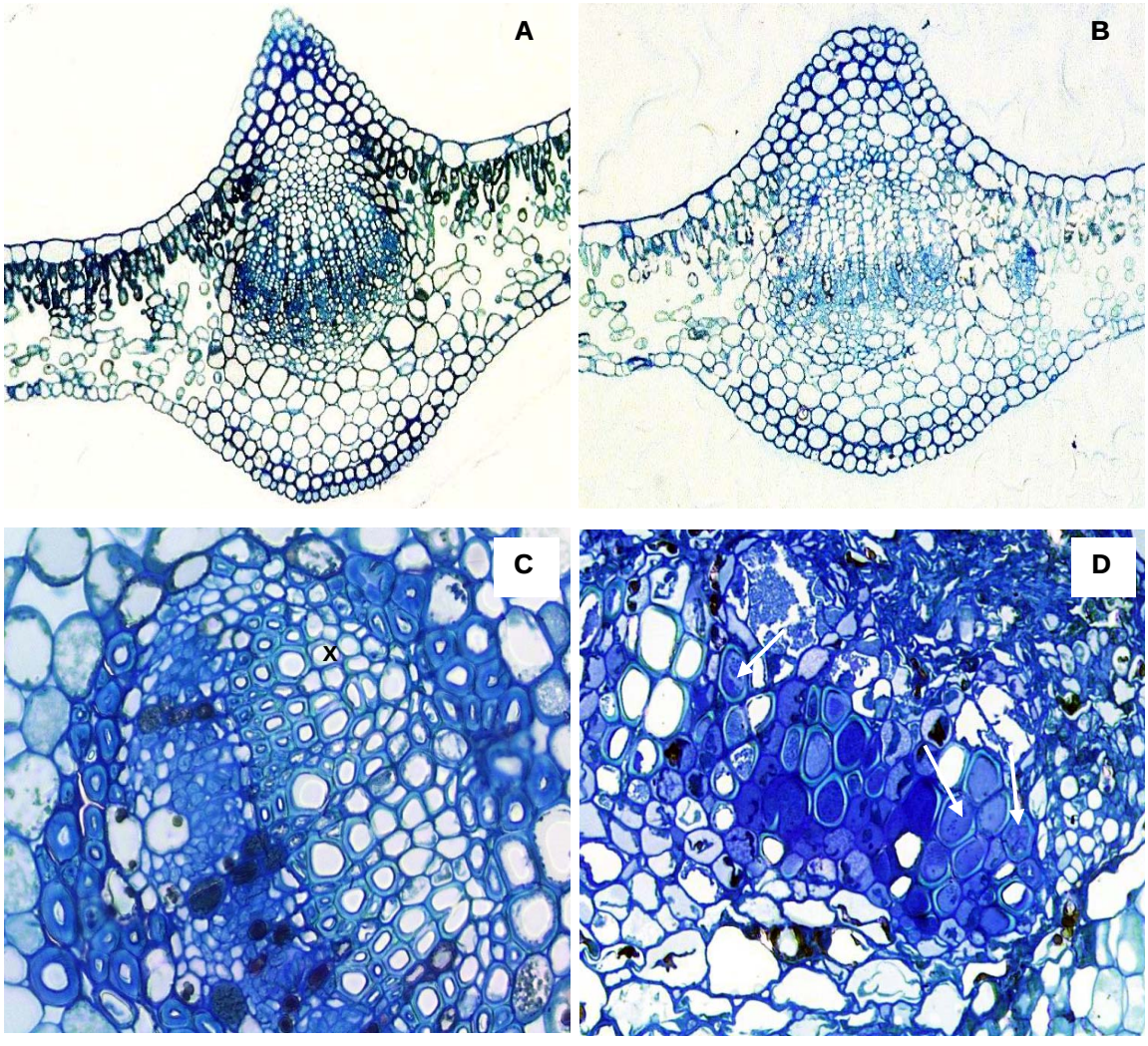


Figure 3.10. Methylene blue-stained light micrographs of cross sections of cv. Conference pear leaf after benzothiadiazole treatment (benzothiadiazole was sprayed 7 and 2 days before pathogen inoculation). **A:** Two days after *E. amylovora* inoculation, **B:** four days after pathogen inoculation, not important tissue structure disorganization was observed (x214), **C:** higher magnification of vein zone of non-inoculated control, the lumen of xylem vessels was entirely clear, and **D:** higher magnification of vein zone four days after bacteria inoculation, the lumen of xylem vessels showed some stained substance and some particles (white arrows), some degree of structural disorganization was also observed (x428). X: xylem.

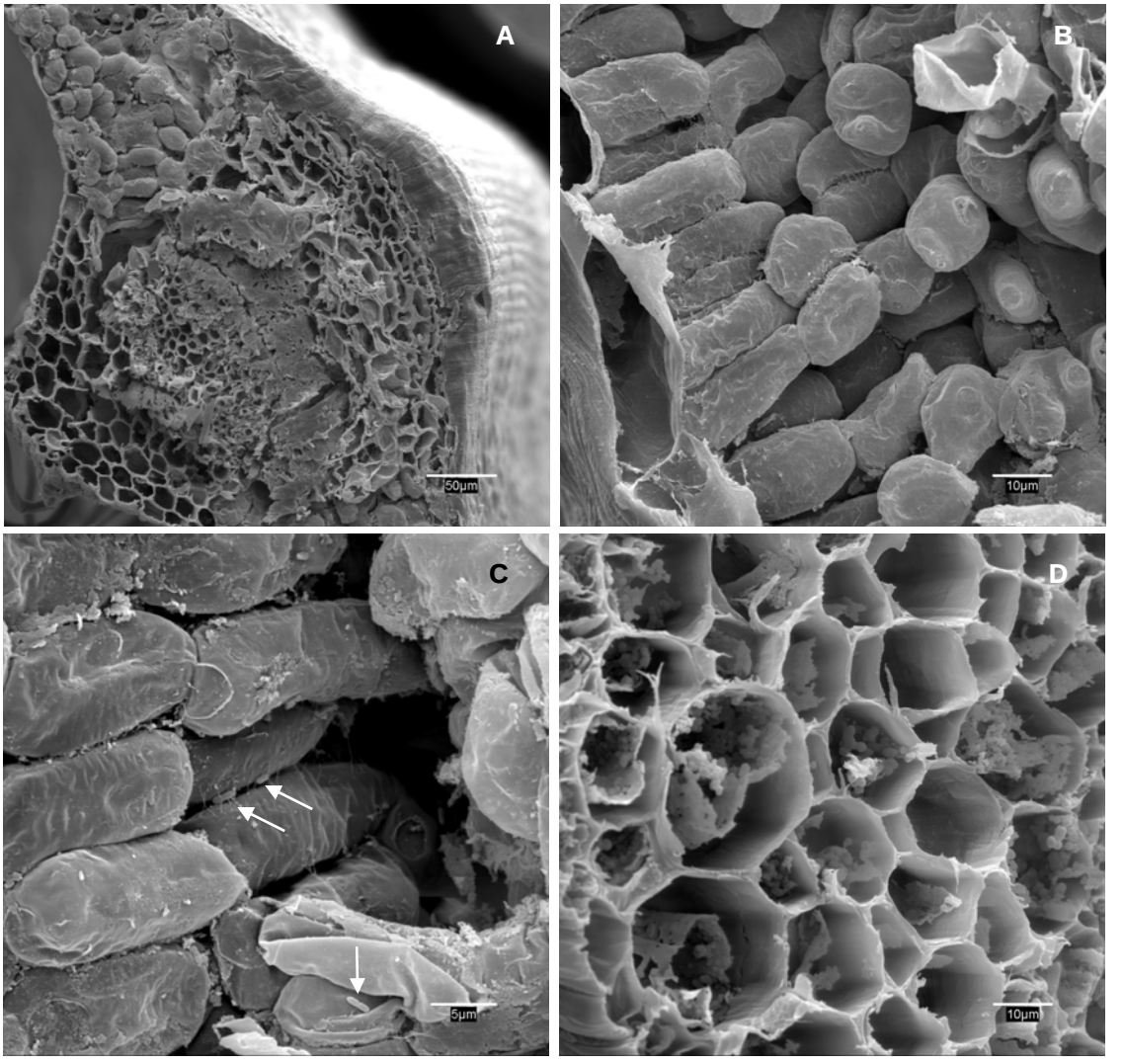


Figure 3.11. Scanning electron micrographs of cv. Conference pear leaf tissue sprayed with benzothiadiazole. **A:** General view of vein zone of non-inoculated control, **B:** palisade parenchyma cells two days after bacterial inoculation, no bacterial cells were observed, **C:** palisade parenchyma cells four days after bacterial inoculation; (white arrows: bacterial cells), and **D:** vein parenchyma cells four days after bacterial inoculation, higher amount of bacterial cells were observed in the lumen of vessels.

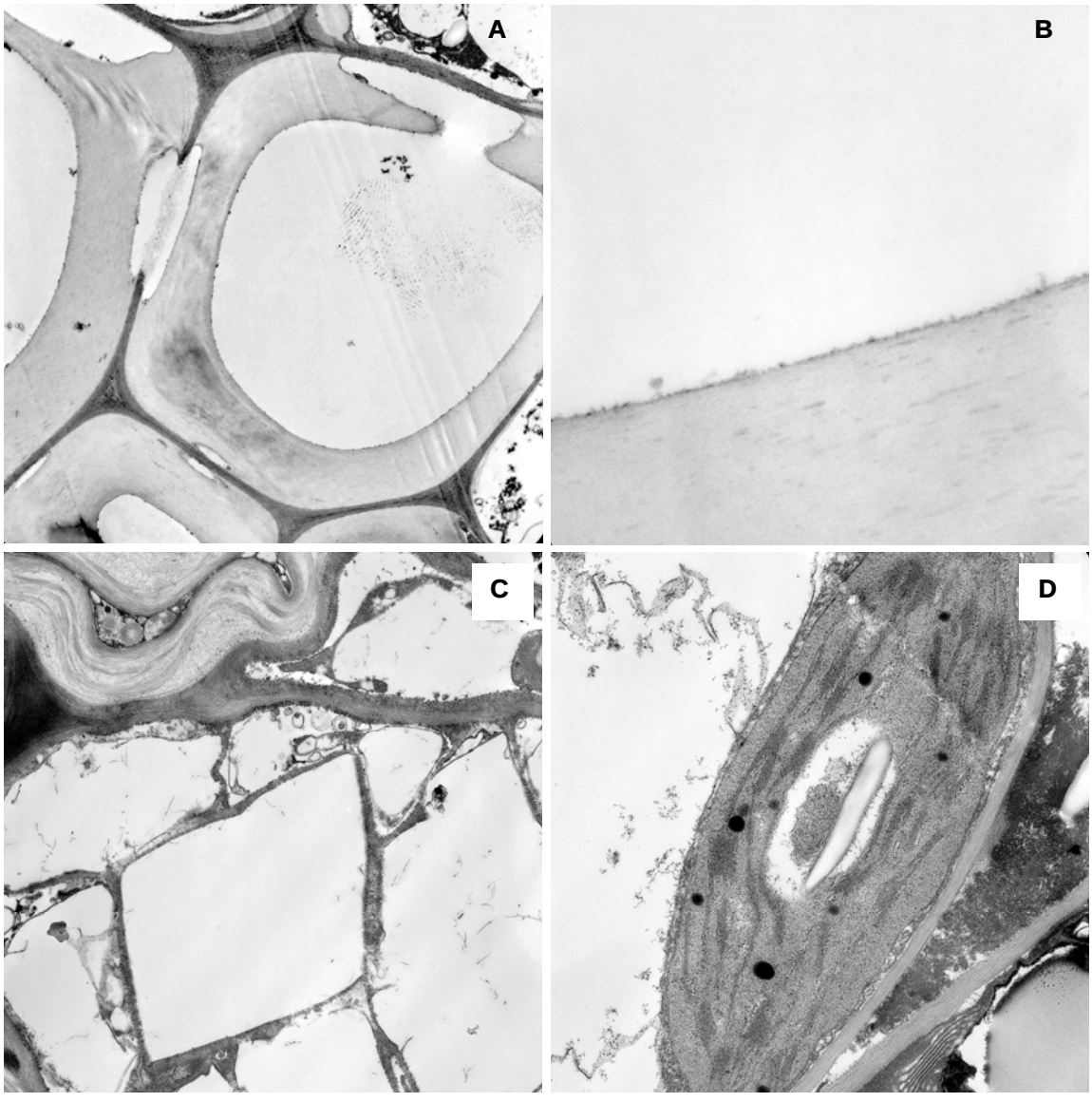


Figure 3.12. Transmission electron micrographs of cv. Conference pear leaf tissue treated with benzothiadiazole and non-*E. amylovora*-inoculated. **A:** The lumen of xylem vessel was clear (x8,000). **B:** The surface of secondary thickening was smooth (x63,000). **C:** cytoplasmic space of parenchyma cells did not show important changes (x10,000), and **D:** higher magnification of a chloroplast without maintaining its integrity (x16,000).

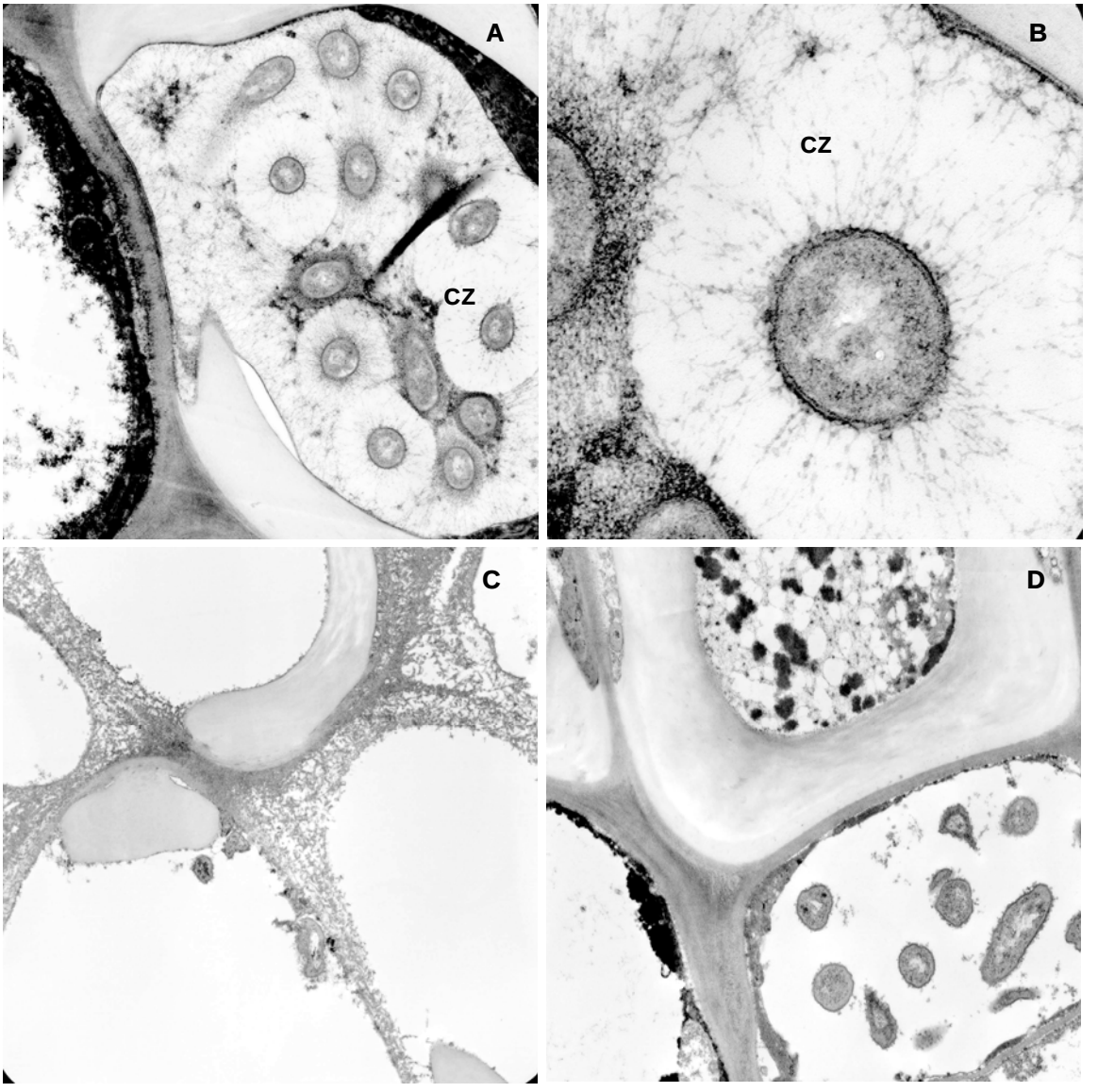


Figure 3.13. Transmission electron micrographs of pear leaf tissue treated with benzothiadiazole two (A,B) and four (C,D) days after *E. amylovora* inoculation. Leaf section 2 mm from the inoculation point. **A:** The xylem vessel contains several bacterial cells surrounded by a well defined clear zone (CZ) without occluding substance (x12,500). **B:** Higher magnification of the clear zone around bacterial cell (x63,000). **C:** Xylem vessels with few bacterial cells. Cell wall seems degraded, only secondary thickening maintain its structure (x8,000). **D:** Electron-dense particles embedded in a granular matrix bordering a xylem parenchyma cell with bacterial cells inside (x10,000).

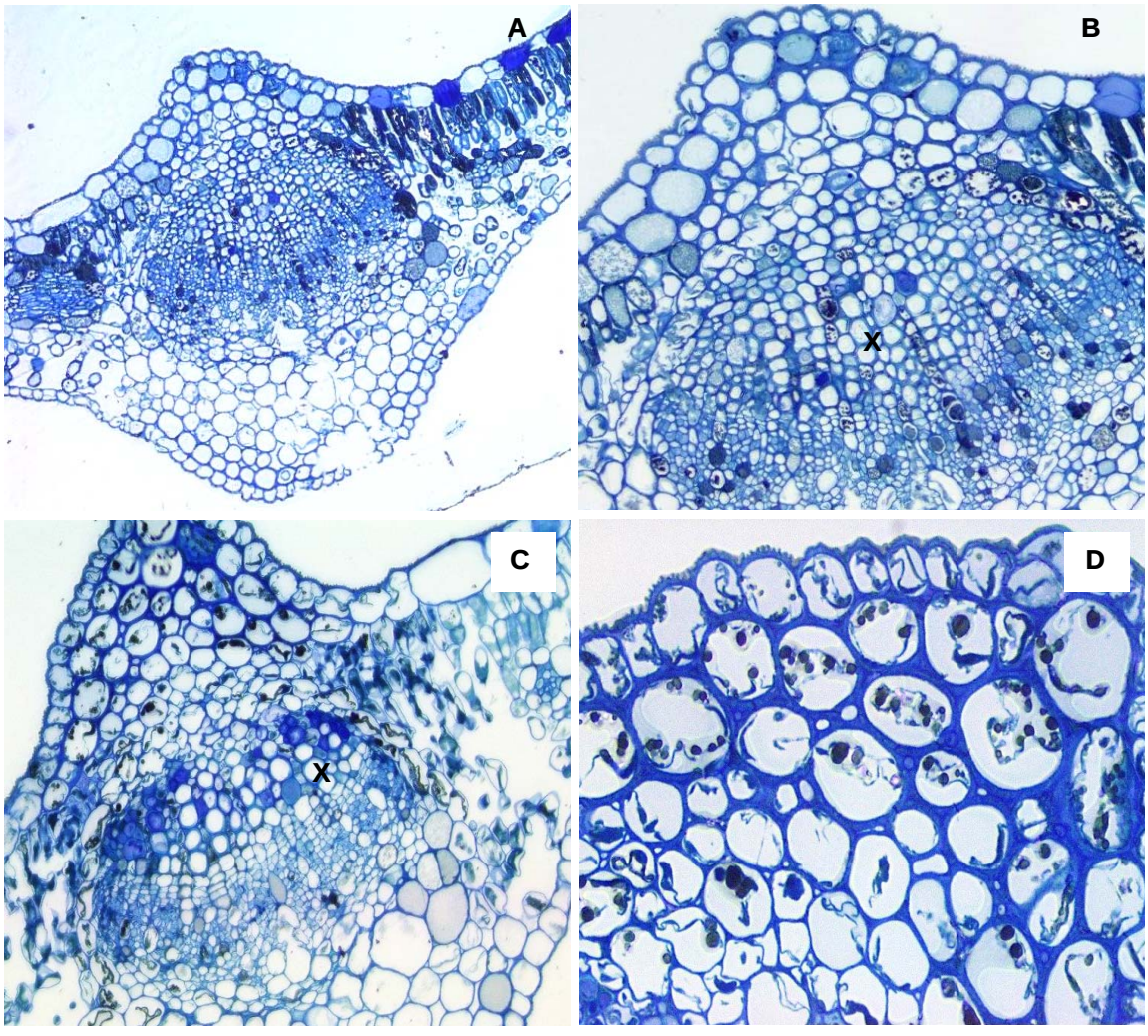


Figure 3.14. Methylene blue-stained light micrographs of cross sections of cv. Conference pear leaf after phosphonate treatments. Phosphonate derivatives were sprayed 7 and 2 days before pathogen inoculation. Observations were performed four days after *E. amylovora* inoculation. **A:** General view of leaf after ethephon sprays (x214), **B:** higher magnification of the vein zone with stained substance inside the xylem vessels (x214), **C:** leaf treated with fosetyl-Al, the lumen of xylem vessels was entirely clear, and **D:** higher magnification of cytoplasmic disorganization of parenchyma cells sprayed with fosetyl-Al (x428). X: xylem.

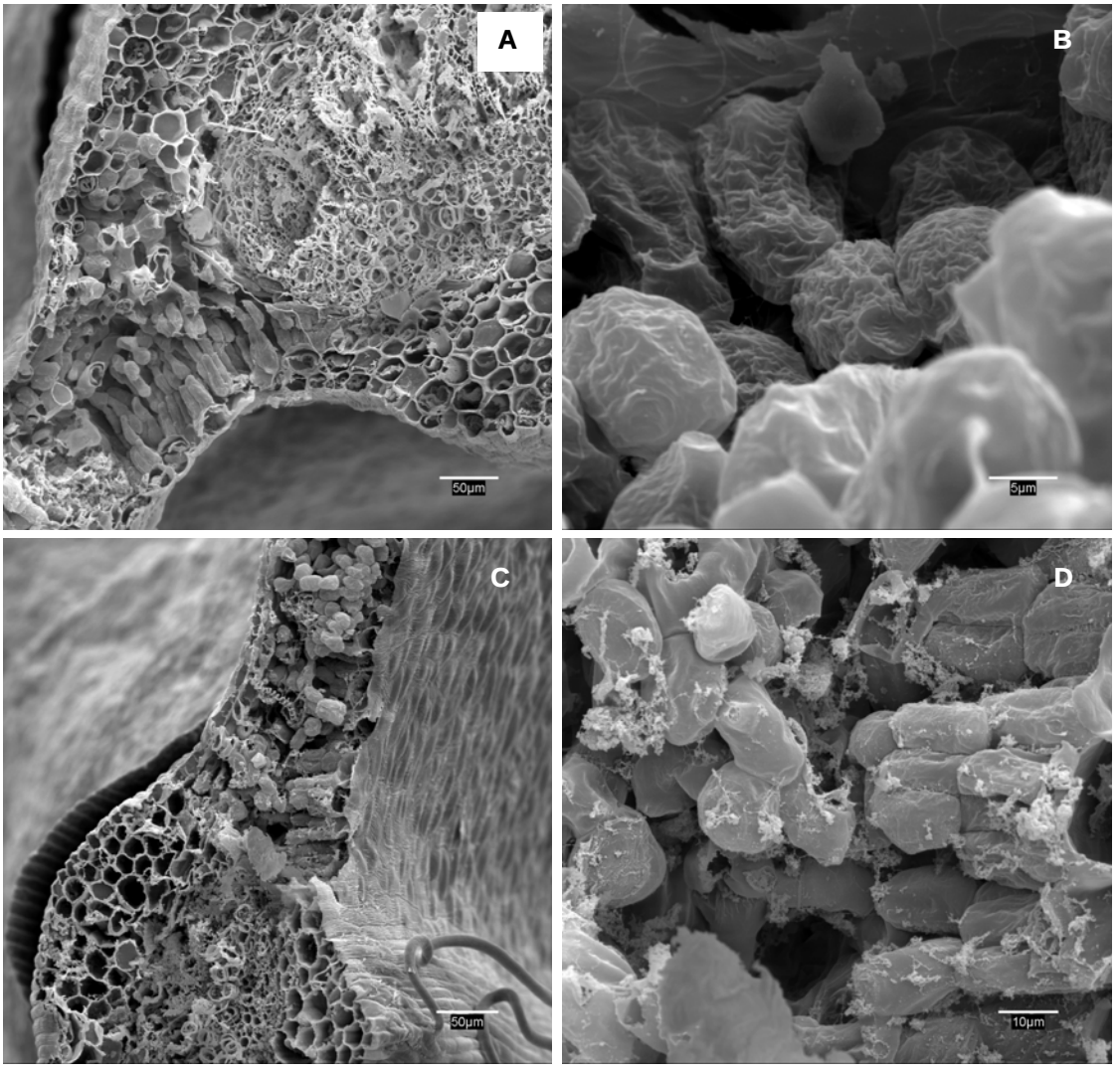


Figure 3.15. Scanning electron micrographs of cv. Conference pear leaf sprayed with phosphonates; ethephon (A and B) and fosetyl-Al (C and D). **A:** General view of vein zone of non-inoculated control, **B:** palisade parenchyma cells four days after bacterial inoculation, no bacterial cells were observed, **C:** fosetyl-Al sprayed plants of non-bacterial-inoculated control, and **D:** parenchyma cells four days after bacterial inoculation, bacterial cells were not observed, and some amorphous substance was seen between parenchyma cells.

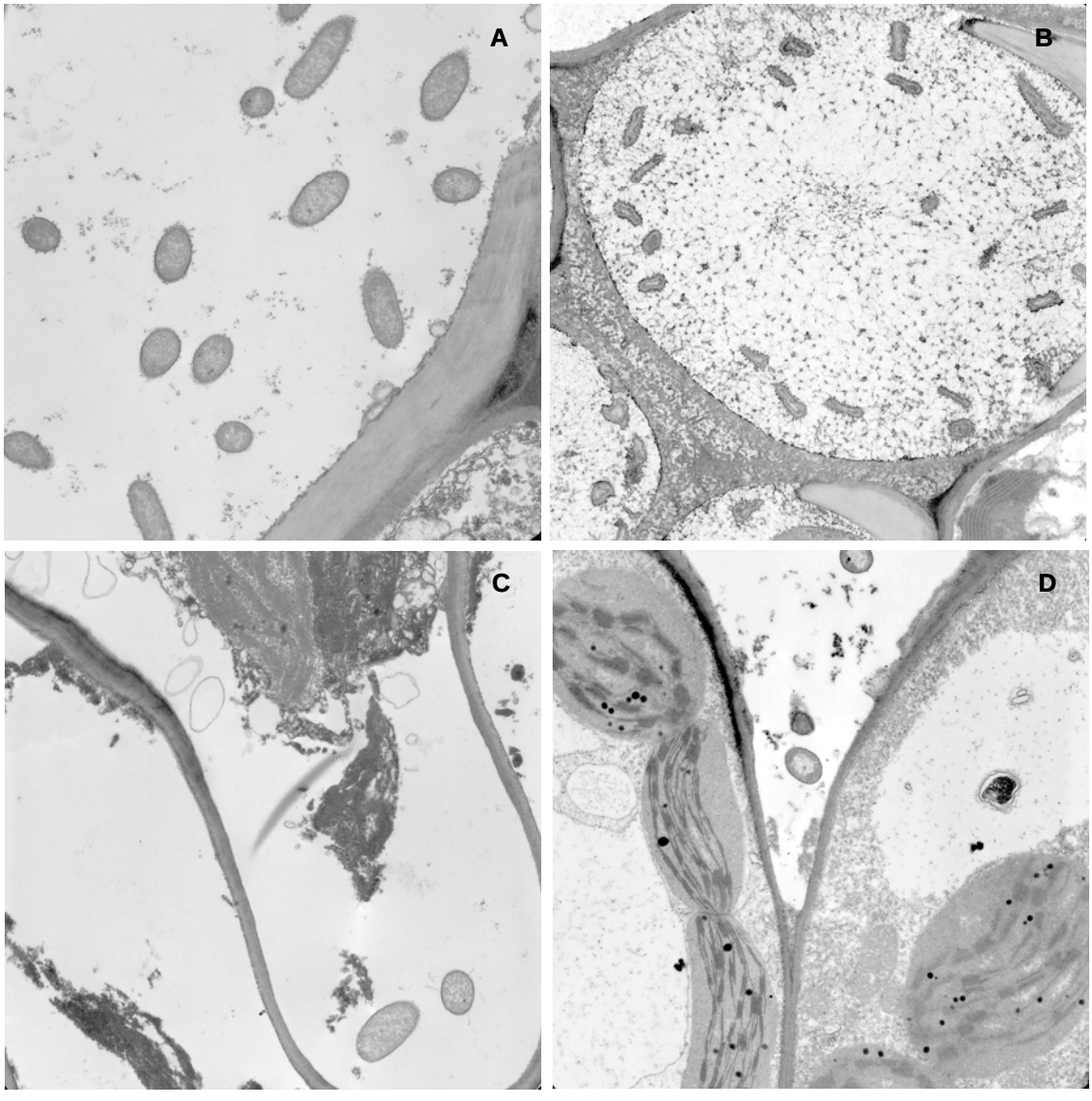


Figure 3.16. Transmission electron micrographs of pear leaf tissue from ethephon-sprayed plants four days after *E. amylovora* inoculation, 2 mm from the inoculation site. **A** and **B:** Bacterial cells inside the xylem vessel (x8,000) (x6,300). **C:** Two bacterial cells inside one parenchyma cell, with some degree of cytoplasmic cell disorganization (8,000). **D:** Presence of bacterial cells in parenchymatic intercellular space (x8,000).

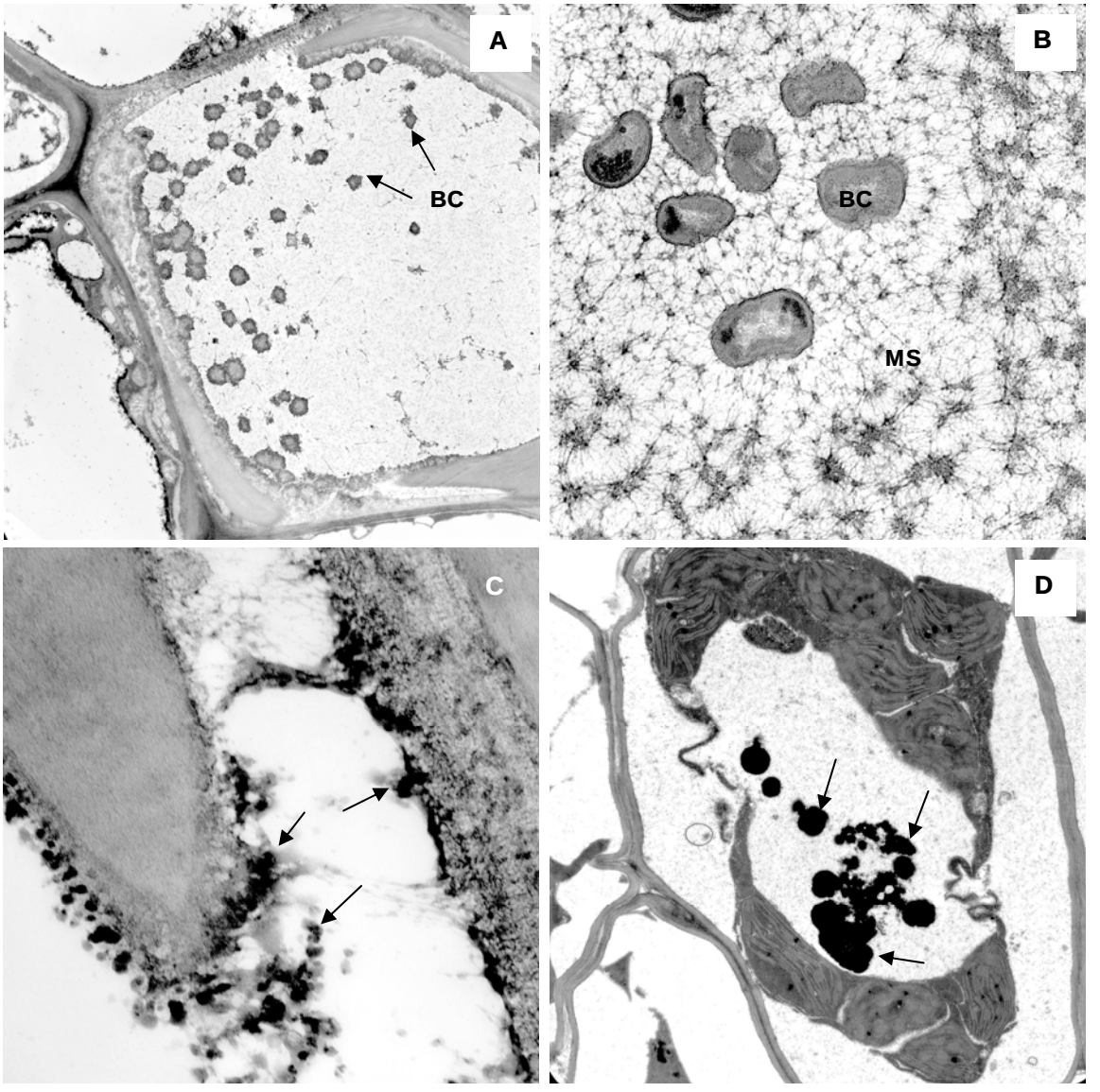


Figure 3.17. Transmission electron micrographs of fosetyl-Al-sprayed pear leaf tissue four days after *Erwinia amylovora* inoculation, 2 mm from the inoculation site. **A:** Xylem vessel containing bacterial cells (BC) (x5,000). **B:** Bacterial cells and matrix substance (MS) (x20,000). **C:** Electron-dense appositions on the surface of secondary wall of xylem vessel (arrows) (x50,000). **D:** Parenchyma cells with cytoplasmic disorganization and electron-dense substance inside (arrows) (x6,300).

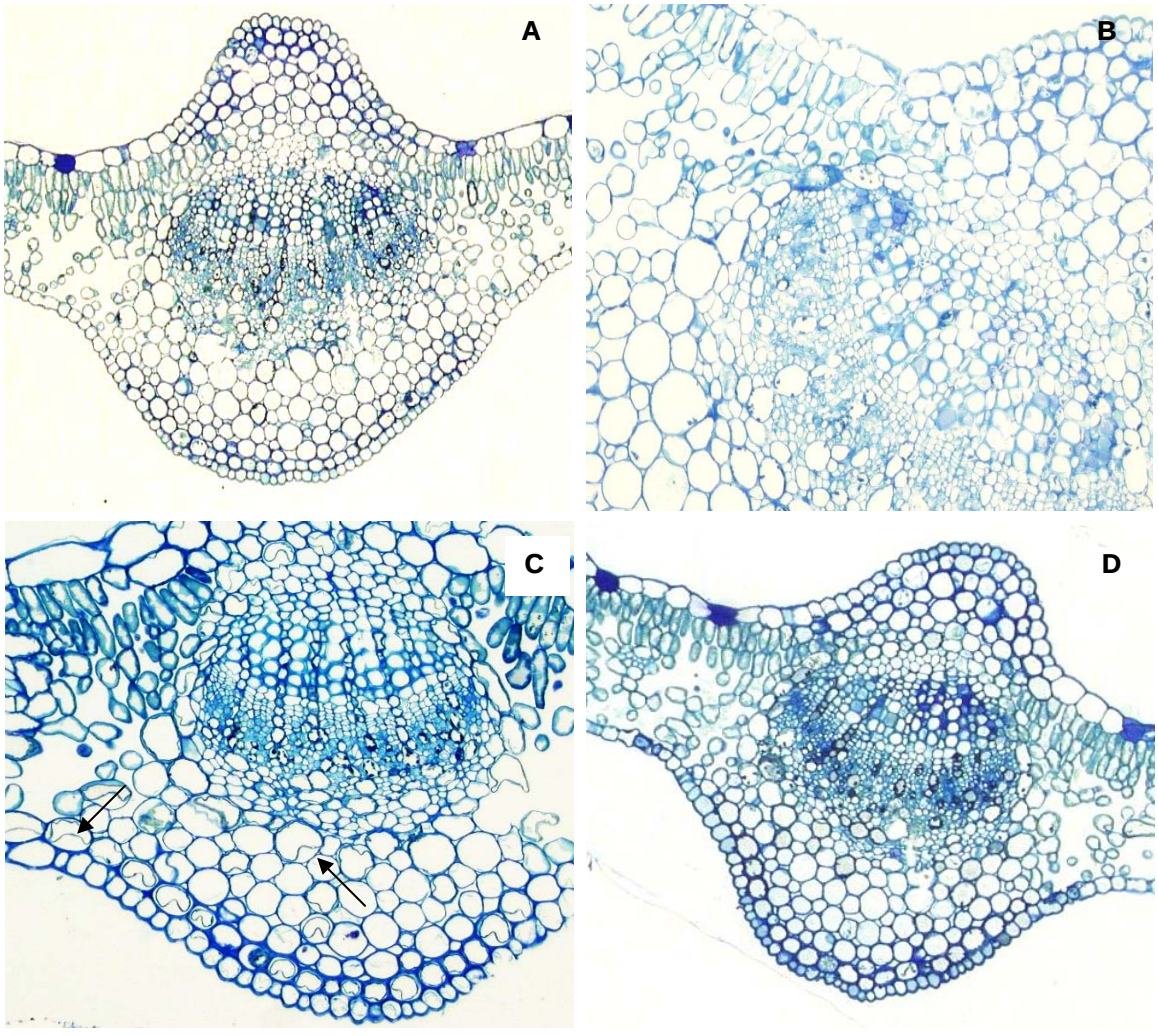


Figure 3.18. Methylene blue-stained light micrographs of cross sections of cv. Conference after copper sulfate and streptomycin application. Chemicals were sprayed 2 hours before pathogen inoculation. **A:** Leaf section obtained two days after *E. amylovora* inoculation of copper sulfate sprayed plants, **B:** copper treated leaf observed four days after pathogen inoculation, higher magnification of vein zone (x214), **C:** streptomycin treated plants without pathogen inoculation, the lumen of xylem vessels was entirely clear, although parenchyma cells had lost their turgidity (arrows), and **D:** streptomycin sprayed plants observed four days after *E. amylovora* inoculation, the lumen of xylem vessels showed some stained substance and some particles (x428).

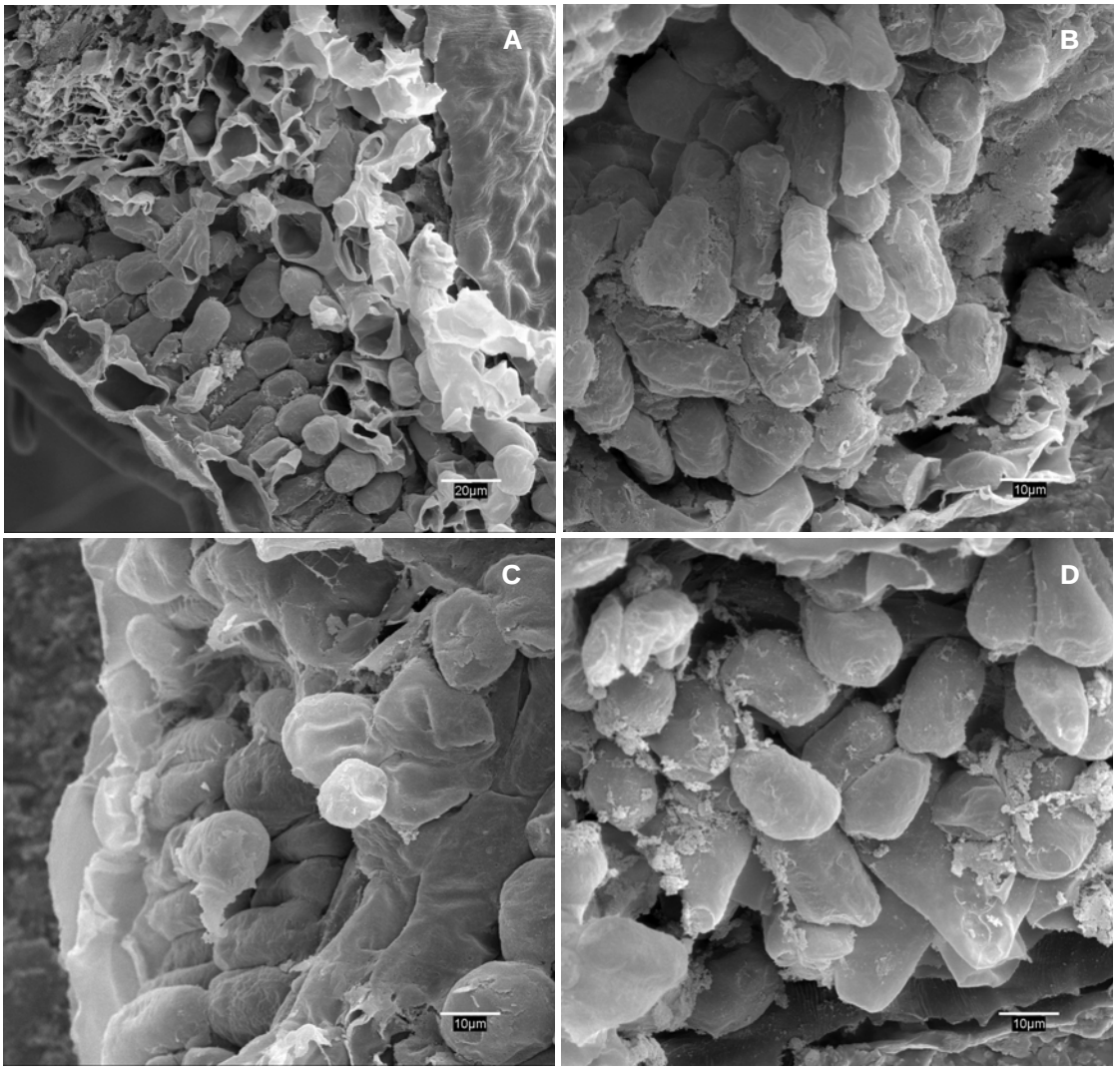


Figure 3.19. Scanning electron micrographs of cv. Conference pear leaf sprayed with copper and streptomycin. **A:** non-bacteria-inoculated and copper-sprayed plants, **B:** palisade parenchyma cells two days after bacterial inoculation in copper treated plants, no bacterial cells were observed, **C:** palisade parenchyma cells of non-*E. amylovora*-inoculated and streptomycin-sprayed plants, and **D:** parenchyma cells four days after bacterial inoculation in streptomycin treated plants, bacterial cells were not observed.

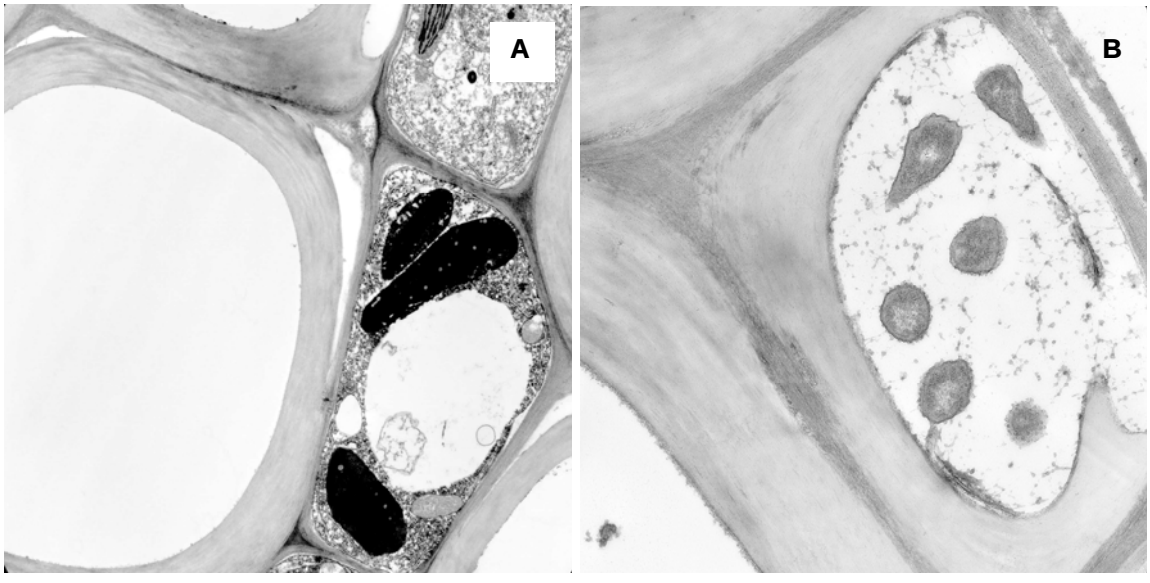


Figure 3.20. Transmission electron micrographs of copper sulfate-sprayed pear leaf tissue. **A:** non-*E. amylovora*-inoculated control (x6,300). **B:** four days after bacterial inoculation (x16,000).

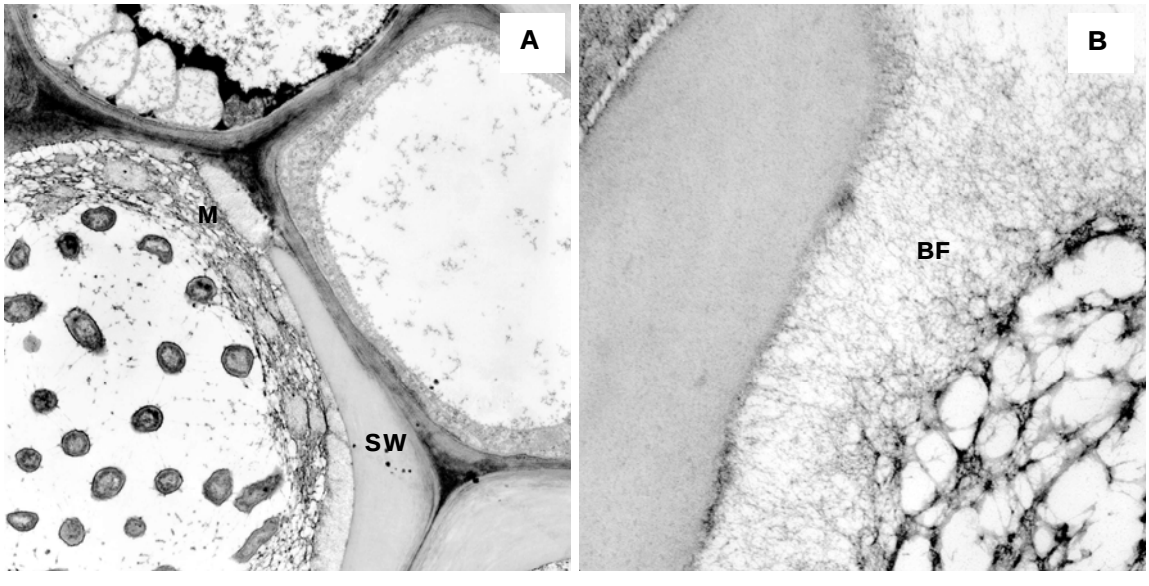


Figure 3.21. Transmission electron micrographs of pear leaf tissue sprayed with streptomycin. **A:** Xylem vessel, four days after bacterial inoculation, a matrix (M) is formed around the secondary wall (SW) (x8,000). **B:** Higher magnification of brushlike fibrils (BF) on the surface of secondary wall of xylem vessel infected, and detail of matrix structure (x50,000).

3.3. MOLECULAR STUDIES OF PEAR LEAVES IN RESPONSE TO BENZOTHIADIAZOLE AND ETHEPHON APPLICATION

Preliminary studies on molecular basis of pear leaf responses to plant defense activators were performed. Expression of defense pathogenesis-related proteins was analyzed one and five days after chemical sprays.

3.3.1. Expression of defense genes

Temporal expression of defense PR genes was analyzed through reverse transcription-polymerase chain reaction (RT-PCR) in leaves of pear plants sprayed with benzothiadiazole, ethephon and water. No transcript accumulation of defense genes was detected in water control except in phenylalanine ammonia lyase (PAL) which was expressed in all sprays, while no induction of PR-5 was detected (data not shown) (Figure 3.22). Ethephon caused a slow induction of β-1,3-glucanase activity detected 5 days after sprays. Benzothiadiazole sprays induced chitinase (CHT) expression 5 days after application. To check that PCR product was the chitinase it was sequenced (Figure 3.23). High homology between the class II chitinase from *Malus x domestica* with accession number (AF494395) and putative chitinase mRNA sequence of pear were observed. These results indicated that it correspond to the same gene.

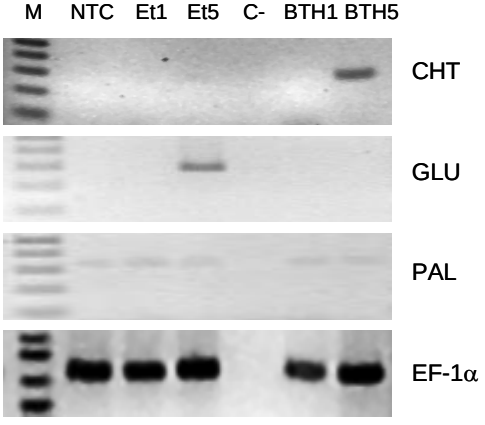


Figure 3.22. Chitinase (CHT), β-1,3-glucanase (GLU), L-phenylalanine ammonia-lyase (PAL) gene expression in pear leaves in non-treated control (NTC), ethephon (Et) and benzothiadiazole (BTH) sprayed plants. RNAs were sampled 1 and 5 days after sprays. Reverse-transcription polymerase chain reaction-derived amplification products were separated on Tris-borate-EDTA agarose gels and stained with ethidium bromide. EF-1α = constitutive elongation factor 1α gene. Experiments were repeated twice. Lane M represents marker. Lane C- represents the control, in which the RT reaction mix without reverse transcriptase was used as a template in the reaction.

```
          1                                                    50
AF494395  GGTCAAACTT CTCATGAAAC TACTGGAGGA TGGGCAAGTG CACCAGATGG
     CHT
          51                                                  100
AF494395  TCCTTATGCA TGGGGATATT GCTTTGTCAA TGAAAGAAAC CAAGATGTGT
     CHT                                                   TGTG-
          101                                                 150
AF494395  ATTGCACACC ATCCGGTCAA TATCCATGTG CTGCTGGCAA GAAATACTAT
     CHT  ATTGCACACC ATCCGGTCAA TATCCATGTG CTGCTGGCAA GAAATACTAT
          151                                                 200
AF494395  GGCAGAGGAC CCATCCAACT TACCCACAAC TACAACTATG GTCAAGCGGG
     CHT  GGCAGAGGAC CCATCCAACT TACCCACAAC TACAACTATG GTCAAGCGGG
          201                                                 250
AF494395  CAAGGCAATC GGAAATGATC TGATAAAAAA CCCGGATCTA GTGGCCACGG
     CHT  CAAGGCAATC GGAAATGATC TGATAAAAAA CCCGGATCTA GTGGCCACGG
          251                                               297
AF494395  ACCCGGTTGT ATCATTCAAG ACAGCTATAT GGTTCTGGAT GACCCC
     CHT  ACCCGGTTGT ATCATTCAAG ACAGCTATAT GGTTCTGGAT GACCCCA
```

Figure 3.23. Comparison of the class II chitinase (CHT-1) from *Malus x domestica* (AF494395) and putative chitinase mRNA sequence of pear (CHT). Red letters correspond to the coincident sequence and black letters the non-coincident sequence.

4. DISCUSSION

Our results indicated that the early site of tissue colonization by *E. amylovora* after pear leaf inoculation was the vascular system rather than intercellular space according to previous works (Crosse *et al.,* 1972; Suhayda and Goodman, 1981b). Light microscopy showed some stained substance and small size particles that could be bacterial cells located mainly in the xylem vessels. Occlusion of the vascular elements by bacteria was more pronounced four days after infection, and coincided with the clearly distinguishable symptoms of wilt observed in inoculated leaves. The data suggested that wounding of leaves exposes the xylem vessels to the pathogen, and this could be an important factor in the infection process in nature as Suhayda and Goodman (1981a) suggested. According to Berger *et al*. (1996) the growth of population inside the plant is unlimited and influenced by temperature, humidity and resistance of cultivar.

Scanning electron microscopy revealed that *E. amylovora* cells were found in two main locations: in the intercellular spaces of the cortical parenchyma of the midrib; and in vascular elements of the midrib according to what has been described previously (Dellagi *et al.,* 1999; Perino *et al.,* 1999). We observed a rapid multiplication of bacteria that took place in the leaf, mainly in and around the vascular tissues from two to four days after inoculation.

Electron micrographs showed that although no external symptoms were visible within two days, ultrastructural alterations, were detected. These included deposition of a vessel-occluding ground substance, the appearance of brushlike fibrils on the inner surfaces and spiral secondary thickenings of the xylem cell walls. Four days after bacteria inoculation, the vessel-occluding substance appeared in more vessels and become denser, in agreement with observations of Goodman and White (1981). Two and four days after *E. amylovora* inoculation the xylem vessels contained some occluding substance as well as bacterial cells. Occasionally this vessel-occluding substance could be seen in xylem vessels of inoculated leaves even though no bacteria were visible. It was possible to observe the xylem vessels or cells bordering the vessels whose lumens did not contain any bacteria but revealed the presence of occluding ground substance. Probably these symptoms reflect the presence of bacteria either above or below the thin section of leaf processed as bacteria were seen in subsequent serial sections, as observed by Goodman and White (1981). Vessels in an advanced state of infection were totally occluded by fibrous material inside which bacteria were clearly distinguishable. The observation of a fibrillar brushlike structure, that appeared more clearly on vessel walls and their thickenings, before these vessels contain high amount of bacteria or ground substance inside them, had been reported for first time by Goodman and White (1981). The origin of the fibrous material is not known, could be of bacterial origin or partially elaborated by the plant. According to Goodman and White (1981) this structure appears to be of host cell origin, since is mainly located in xylem vessel walls and their thickenings.

The release of bacteria from the xylem and subsequent plasmolysis of parenchyma cells was previously documented by electron microscopy (Goodman and White, 1981; Bogs *et al.,* 1998). Our experiments demonstrate that bacteria can colonize the intercellular spaces of the parenchyma after disrupting the xylem vessels. Movement of *E. amylovora* from the xylem into the parenchyma was not restricted to the site of wounding, as described by Suhayda and Goodman (1981b). The disintegration of cell walls observed in histological studies may have been due to a mechanical rupture caused by bacterial masses, since it has been demonstrated by Seemüller and Beer (1976) that *E. amylovora* did not produce cell-wall degrading enzymes. Their analyses indicated that the fire blight pathogen did not produce, *in vivo* or *in vitro*, detectable levels of any of the three most prevalent classes of polysaccharide degrading enzymes; pectinases, cellulases, or xylases. According to Youle and Cooper (1987) the presence of bacteria in the intercellular spaces induces dysfunction of host cell membranes, resulting in ion and nutrient leakage.

As we observed the sequence of events in *E. amylovora* infection was, first the bacteria began to multiply in a limited number of vessels near the inoculation point, which showed early stages of lumen occlusion. Neighboring vessels may be free of both bacteria and ground substance; however, the cell wall and surface of their spiral secondary thickening were covered by fibrils that had a brushlike orientation. This was probably an early stage of occluding ground substance formation. Probably, these fibrils had developed in the xylem vessels in response to the presence of bacteria in the lumen of the neighboring vessel elements. Later, as the number of bacteria in vessels increased and the numbers of vessels containing bacteria also increased, the fibrillar

orientation changed to a granular configuration in vessels where bacteria were present or nearby. As reported by Goodman and White (1981), the increased density of occluding substance and the transformation of the fibrils to a granular form may reflect the aggregation of these two components into a gellike structure, which seemed identical in appearance with gels described by Suhayda and Goodman (1981b) that were induced either by amylovoran or *E. amylovora per se*.

The spectrum of activity of BTH has to be worked out for each system, each host/pathogen-plant activator interaction is unique. BTH-activated plants are often able to defend themselves against a broad spectrum of diseases (Tally *et al*., 1999). A main host response in the BTH-induced resistance responses was the occlusion of xylem vessels with an amorphous material. In non-inoculated benzothiadiazole-treated plants no evidence of cell-wall reinforcement, previously described in other annual species (Benhamou and Bélanger, 1998), was observed after ultrastuctural studies. This is probably due to two reasons; the lack of a method that allows us to accurately determine these changes as inmunocytochemical labeling, and the possibility that in perennial plants the time required for the cell-wall reinforcement is longer than in annual plants (four days are not enough to observe these changes in pear plants). However, in *E. amylovora*-inoculated and BTH-sprayed plants several changes were observed either two or four days after infection. Two days after infection in BTH-treated plants a well-delimited clear zone around the bacteria was observed, which was not observed in other chemical treatments and which was not previously described by other authors. It is remarkable the clear zone that only was observed two days after infection, four days after inoculation this clear zone disappeared. Four days after infection no host-cell disorganization was observed but in comparison with the non-BTH-treated leaves a delaying infection was observed.
Moragrega (1997) observed in ethephon-treated and *Pseudomonas syringae* pv. *syringae*-inoculated pear leaves that bacteria cells were surrounded by fibrillar material while plant cells showed some disorganization and lost their turgidity, and also that this changes were more evident than in fosetyl-Al-treated plants. According to this work, results of the present ultrastructural study with *E. amylovora*-inoculated plants support the idea that some cell disorganization is observed either in ethephon-treated or fosetyl-Al-treated plants.

Accumulation of PR proteins is one of the most common markers of active plant defense. Among them the hydrolytic enzymes β-1,3-glucanases (GLU) and chitinases (CHT) have been suggested to be involved in apple resistance to fire blight pathogen (Brisset *et al.,* 2000). In pear there is a lack of complete understanding of induction of PR proteins upon pathogen challenge or chemical defense-induction treatment. Extraction of RNA from pear leaves is not as easy as from apple, as we have experimented and Venisse *et al*. (2002) suggested. GLU, CHT, and PR-5 have been described as having mainly antifungal activities, although some chitinases can also display lysozyme activity and are able to hydrolyze bacterial peptidoglycan (Fritig *et al*., 1998). Results obtained here show that after BTH application, pear untreated-leaves (upper-leaves in plant)

expressed one PR-protein, CHT. This result contrasts with those obtained by Ziadi *et al*. (2001) who observed that chitinase activity was not induced by BTH in cauliflower, and agree with those from Lawton *et al*. (1996) and Wendehenne *et al.* (1998) who reported that BTH induced the accumulation of acidic-PR-protein and PR-3 mRNAs in *Arabidopsis thaliana* and tobacco, respectively. β-1,3-glucanases expression was not systemically induced by BTH in pear while ethephon applied five days before analysis, showed some expression, in contrast with previous results by Brisset *et al*. (2000). We cannot exclude the possibility that other isoforms of this gene (not detected with our probes) are induced by BTH. CHT and GLU are particularly useful markers for SAR, since they were not expressed in non-treated controls, in contrast, with the constitutive expression of PAL in either induced or non-induced plants. Finally PR-5 was not systemically expressed either in chemical-induced treatments or non-treated plants, in contrast with high PR-5 transcript expression observed by Venisse *et al*. (2002) in flowers and shoots of apple after *E. amylovora* infiltration.

We can conclude that, although weak, an evidence is provided in this study suggesting that BTH has the ability to induce SAR in pear. Exogenous foliar application of the chemical stimulated the plant defense reactions to culminate in the establishment of a toxic environment for the pathogen, and in the elaboration of permeability barriers. The possibility of inducing a plant to respond more rapidly and more efficiently to pathogen attack by prior treatment with selected chemicals can be considered as one of the most encouraging options for effective management of plant diseases including fire blight in the near future.

Based on the present results, the defense strategy occurring at the onset of BTH-mediated induced resistance in pear plants appears to follow a specific scheme of events, including (a) the delay of the pathogen growth probably by reinforcement of host-cell wall, and (b) the activation of host secondary responses with antimicrobial activity.

These results indicates that the response of pear leaves to the elicitor treatment is similar to that observed in other plant-elicitor interactions and suggests the involvement of pear biochemical defense responses in plant defense activators-mediated disease resistance.

The introduction of the commercial plant defense activator, acibenzolar-S-methyl (ASM, Bion®; Kunz *et al*., 1997) provides a new option for chemical management of plant diseases based on the induction of host resistance. The mechanism of induction, signal pathways, and expression of induced resistance are being intensively studied in annual crops and genetic model such as *Arabidopsis* (Hammmerschmidt, 1999). Less well known is the nature and expression of such systemic effects in woody perennial plants.

Finally, the knowledge about the ultrastructural changes during the *E. amylovora* infection can improve the control methods of disease. However, further research will be necessary in order to understand how acibenzolar-S-methyl triggers the induction of resistance.

CHAPTER 4

DECISION SUPPORT SYSTEM FOR FIRE BLIGHT RISK IN SPAIN

1. INTRODUCTION

1.1. Decision support systems

A decision support system (DSS) is an interactive computer program that utilizes analytical methods, such as decision analysis, optimization algorithms, program scheduling routines, and so on, for developing models to help decision makers formulate alternatives, analyze their impacts, and interpret and select appropriate options for implementation (Adelman, 1992). Another definition includes any and all data, information, expertise or activities that contribute to option selection (Andriole, 1989). An explicit common idea of these definitions is that DSS integrate various technologies and aid in option selection. There is general agreement that DSS focus on decisions and on supporting rather than replacing the user's decision-making process (Keenan, 1997). A new generation of DSS has been developed, based on mathematical models which simulate the development of crops and their associated pests and diseases, accounting for the impact of weather, pest pressure and other factors, and predicting the outcome of possible remedial measures such as application of pesticides (Figure 4.1).

1.2. Plant disease models

A plant disease model is a mathematical description of the interaction between environmental, host, and pathogen variables that can result in disease. A model can be presented as a simple rule, an equation, a graph, or a table. The output of a model can be a numerical index of disease risk, predicted disease incidence or severity, and/or predicted inoculum development (University of California, 2003). They can be used to predict disease risk and development, as a warning system, and ultimately aid in the timing of pesticide applications (Llorente *et al*., 2000). Input variables and/or other parameters such as timing of model initiation may need adjustment due to pathogen biology, host phenology, and variety in a specific area. Plant disease models are typically

developed in specific climates and regions around the world and may contain assumptions about site specific conditions that might not apply for all areas. Therefore, before using a model that was not field tested or validated for a specific location, the model should be tested for one or more seasons under local conditions to verify that it will work in this location.

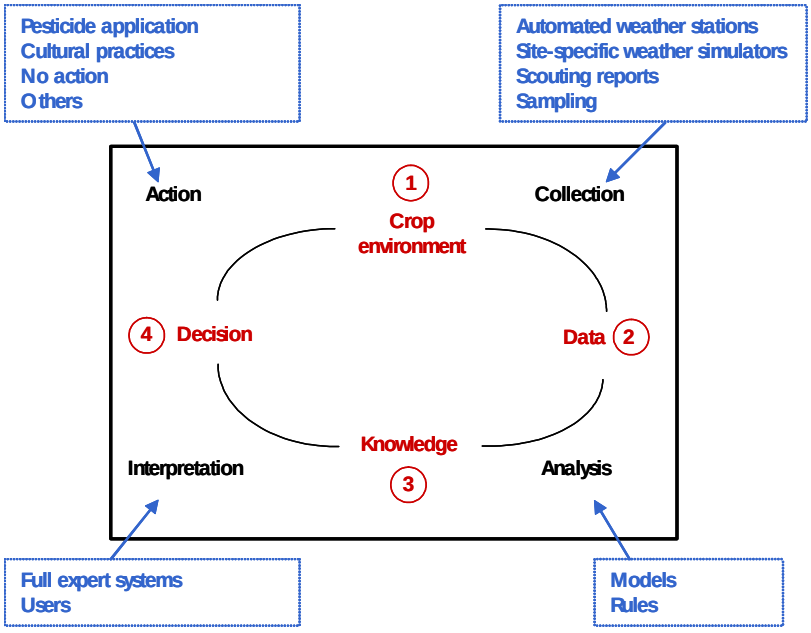


Figure 4.1. Scheme of a decision support system for plant disease management showing components (red), methodology (black), and tools (blue). Modified from Petersen *et al.,* 1993.

1.3. Applications

Warning systems for orchard pest development and associated risk have been developed for many pathogens. Many of these systems require input of current weather data. Use of these predictive models in addition to other information such as phenological stage or susceptibility, can help growers accurately time pesticide applications for the best effect, and avoid spraying when there is no or reduced risk of pest damage. A delayed intervention, in the case of bacterial diseases against which there exist only products of preventive action, is usually ineffective. There is a strong need for local validation of all models, plus development and validation of additional new models. The precision and reliability of any system will depend on the quality and quantity of the input and its relationship with the growth and physiology of the host plant and the pathogen (Billing, 1984). Disease forecasting also allows the prediction of probable outbreaks or increases in intensity of disease and, therefore, allows us to determine whether, when, and where a particular management practice should be applied (Agrios, 1997).

1.4. Fire blight risk forecast models

Several models have been described for fire blight risk prediction, first in North-America and then in Europe. Many of them are based on the principles that: (a) a certain number of heat units, usually in excess of 18.3 °C, must accumulate during bloom period before a threshold level of inoculum has been reached; and (b) wetness is necessary after this point to wash the bacteria to their infection sites (Mills, 1955; Powell, 1965). Several mean temperature and degree-hour models are available to assist in predicting fire blight infection periods and the need for control (Table 4.1). Most of them predict the likelihood of an infection event, and estimate the likely time of the first symptom.

Table 4.1. Fire blight risk predictive models

Model and reference	**Input variables** *Environmental measured and calculated* *Pathogen* *Host*	**Prediction event**	**Stage of model**
Mills (Mills, 1955)	Tmax, rainfall, RH	Risk of blossom blight	Without use
Powell (Powell, 1965)	Tmax, tmin, rainfall, RH	Risk of pre-bloom and blossom blight	Without use
Thomson (Thomson *et al.,* 1975)	Tmax, tmin Mean temperature	Bactericide application	Without use
BOS (Billing, 1980)	Tmax, tmin, rainfall Potential daily doublings of bacteria	Infection period First symptom Risk may be seriously underestimated	Without use
Parafeu-Firescreens (Jacquart-Romon and Paulin, 1991)	Tmax, tmin, rainfall Activity of disease Plant growth and development Inoculum and climatic potential	Infection risk First symptom	Without use Commercially available
BRS (Billing, 1990; Billing, 1992)	Tmax, tmin, rainfall Potential daily doublings of bacteria Bud phenology, tissue damage due to frost or wind	Infection period First symptom	Implementation
Maryblyt (Steiner, 1990)	Tmax, tmin, rainfall, wet Degree-hours, degree-days Bud phenology, damage	Infection risk Level of risk First symptom	Implementation Commercially available
BIS95 (Billing, 1996)	Tmax, tmin, rainfall Degree-days>18 ºC maximum Degree-days>13 ºC mean Blossom periods, tissue damage due to frost or wind New disease and signs of stem invasion	Blossom blight risk First symptom	Implementation
Cougarblight (Smith, 1993)	Wetness, temperature Degree-hours Potential for pathogen presence due to proximity of fire blight cankers Variety, age, vigor, presence of blossoms	Risk of fire blight infection on flower blossom	Implementation
Feuerbra and Anlafbra (Berger *et al.*, 1996)	Tmax, tmin, rainfall Cultivar, phenology	Infection risk First symptom Estimating regional and specific orchard risk	Validation

1.4.1. **Mills' system.** Is based on correlations between temperature and precipitation, and assess the risk of apple and pear blossom blight in the area of Lake Ontario and in western New York (Mills, 1955). The criteria for blossom blight are a daily maximum temperature of 18 ºC after the beginning of bloom, plus precipitation and high humidity (≥70%). Severe blight is predicted with temperature of 24 ºC and precipitation or 27 ºC without precipitation. It is a very simple system, but the information is not very precise.

1.4.2. **Powell's system.** Also use temperature criteria for apple and pear blossom blight and additionally look at the pre-bloom period in Illinois. The criteria for blossom blight are a minimum of 16.5 degree days above 18.3 ºC between latest pre-bloom freeze and early bloom. Severe blight is predicted with temperature from 21-29 ºC and light rain or high humidity (Powell, 1965). It is a system which is easy to use.

1.4.3. **Thomson's system.** It is based on monitoring epiphytic populations of *E. amylovora* to improve the timing of bactericide application for fire blight control (Thomson *et al.,* 1975). Treatment was initiated when the mean temperature exceeded a mean temperature prediction line drawn from 16.7 ºC on 1 March to 14.4 ºC on 1 May. The 16.7-14.4 ºC mean temperature line is a good predictor of when to initiate bactericide applications in pear orchards. Bacteria were detected before the mean temperature exceeded the prediction line only in an occasional orchard. Use of the daily mean temperature to predict the need for bactericide applications will usually result in more bactericide applications than the monitoring technique will. However the temperature technique is simple and inexpensive and can be accomplished by the grower with a recording thermograph or even with a maximum-minimum thermometer (Thomson *et al.,* 1982). However, in some places, a simple relationship between mean temperatures and epiphytic populations was not found. It seemed, therefore, that the approach may not be widely applicable (Billing, 2000).

1.4.4. **Billing's original system (BOS).** Is based on potential doublings (PD), derived from *in vitro* growth rates of *E. amylovora* at different temperatures, combined with rain score from daily precipitation values. From the calculation of theoretical incubation periods, establishment of infection risk and insect risk assessment, the method gives the guidelines for an evaluation of the infection risk over the whole growing season (Billing, 1980). **PD** is the potential doubling of *E. amylovora* in its host according to daily minimal and maximal temperature, when water is not a limiting factor. At moderately warm temperatures in the 18.3-23.9 ^{o}C range, the bacterium has the potential to double every 20-30 minutes. One bacterium gives rise to 1 trillion cells with just 31 divisions which occur within just 2-3 days. The **incubation period** is the time from infection to visible symptoms. Studies of field data from Billing, England, on the duration period of fire blight revealed that temperature and rainfall were positively and interactively correlated with the development rate of fire blight. This model predicts the beginning of the infectious period and the date of appearance of the first symptoms. One of the advantages is that it is graphical in appearance.

Limitations. BOS predicted risk was built on temperature thresholds, relating temperature with fire blight risk (<18 ºC=very low; 18 ºC=low; 21 ºC=moderate; 24 ºC=high; 27-30 ºC=very high) in conjunction with a rain score as a moisture index, but a simple temperature-based system can often prove inadequate for optimal timing of predictive spray application (Billing, 1990). This system included all cool wet days and omitted very warm dry days. Billing's PD values are underestimated, as it takes a longer time for the accumulative PD values to exceed the threshold, and therefore the duration of the incubation period will be overestimated. As the ultimate length of the infection period is overestimated, the risk may be seriously underestimated. In a warning system this may lead to warnings that are too late for effective pruning out of diseased tree parts (Schouten, 1987). However, Billing concluded that infection occurred on fewer occasions than suggested,

probably due to the fact that the model has been evaluated in a zone under low disease pressure. The main problem is that some aspects of BOS were misunderstood and misused by users (Billing, 2000).

1.4.5. **Parafeu** or **Firescreens**. It is a computerized warning system developed in France (INRA). It is based upon the determination at the local level of a climatic potential (CP), and an inoculum potential (IP). CP is determined by data derived from BOS, associated with forecasts of meteorological parameters; IP is estimated mainly from knowledge of the local past history of the disease. These potentials are combined in order to estimate an overall level of risk that allows a decision to be made: no action, visit the orchard to detect and remove symptoms, spray with a suitable chemical (Jacquart-Romon and Paulin, 1991). Estimation of potential inoculum was an essential condition for forecast (Paulin *et al.*, 1994). This system emphasizes the importance of combining good orchard practices with protective spray applications.
Limitations. This warning system is based on BOS and two revised versions have been appeared, and several changes have been made concerning the calculation of PD values. This system must be used at the orchard level, not over large areas such as a production zone.

1.4.6. **Billing's revised system (BRS).** It is a modification of Billing's original system, which was intended to make it simpler, more logical, and more precise (Billing, 1990; Billing, 1992). The main difference with respect to the BOS is that the potential doublings of the pathogen are modified in agreement with the results of Schouten (1987), and that it also considers the influence of trace rain and dry but warm days during bloom period. An infection risk (IR) is calculated with PD values and rain scores. An IR higher than 2 indicates the start of incubation period, which finishes when the sum of PD values is higher than a certain threshold. Later in the season, the Billing system with Schouten's corrections of the PD values can indicate periods with high climatological risk. However, chemical control measures should always be combined with measures to reduce bacterial inoculum in and around the orchard (Deckers *et al.,* 1990).
Limitations. The IR principles seemed useful when blossom blight incidence was high, but not when it was low. This model is intended to be used by people with previous experience and knowledge of the epidemiology of fire blight. BRS aims to give guidance to experienced workers, not to provide rules for action by the inexperienced. Other limitations of BRS, according to Billing, are mainly due to a lack of knowledge of the disease and the quality of the data used as input into the model, problems that are also true for other disease models. It is however worthy of note that other fire blight prediction systems are based on this system.

1.4.7. **Maryblyt.** This is the most used model because it was the first to be commercially available and completely computerized (Steiner, 1990). This system predicts infection risk, level of risk and the appearance of symptoms in different parts of the plant (blossom, shoot, cankers). On the assumption that the fire blight bacteria are present and the plant is susceptible, the following events need to occur in sequence for a fire blight infection to take place:

1. Flowers open with stigmas and petals intact.
2. The accumulation of at least 110 degree hours (a degree hour is one hour for every degree centigrade above the threshold temperature) when temperatures are greater than 18.3 ºC after first bloom.
3. Wetting caused either by dew or 0.25 mm of rain (or at least 2.5 mm of rain the previous day).
4. Average daily temperature of at least 15.6 ºC.
5. All of the above in the sequence shown.

Maryblyt, determined the heat sum required before wetting for significant blossom infection to occur when flowers, colonized by the pathogen, were the source of inoculum. It then determined the heat sum required,

following infection, for development rates, early canker activity, direct and indirect shoot blight and insect activity of insect vectors associated with fire blight (leafhoppers, plants bugs, psylla) (Billing, 2000). Maryblyt is flexible system and can be adapted to local conditions.
Limitations. Van der Zwet *et al*. (1990) compare Maryblyt model with BRS and concluded that comparing years with severe, light, or no fire blight indicated that the two systems were complementary but the specificity of the Maryblyt model appeared more accurate in anticipating infection periods (Steiner, 1990).

1.4.8. **Billing's integrated system, 1995 (BIS95).** It was designed as a substitute for BRS to predict the risk of fire blight in apples, pears and related hosts. It is flexible enough to be applied to all hosts in all climatic areas. The model uses two types of degree-day calculations, which are counted and summed on a daily basis, to help assess the risk of fire blight (Billing, 1996). It incorporates the most useful features of BRS, the Maryblyt model (Steiner, 1990) and earlier approaches by various workers. BIS95 contains novel features which were influenced by the nature of the pathogen and the epidemiology of fire blight which differs markedly from common fungal diseases of top fruit (Berrie and Billing, 1997). In BIS95, for simplicity, degree-day sums are used, pending evidence that degree-hour sums would significantly increase precision. Degree-days above two different temperature thresholds are used: 18 ºC maximum (DD18 max) and 13 ºC mean (DD13 mean). Daily values of 1 ºC or more above 18 ºC max or 0.5 ºC above 13 ºC mean are counted as DD and summed. Sums of DD18 max starting on the first day of bloom, dates when the DD18 max sums reach 17 or multiples (34, 51, 68, 85) are noted as guides to blossom blight risks. Sums of DD13 mean start on the day after each infection risk (IR) day, dates when the DD13 mean sums reach 17 or more (or 47 for apple blossom blight) are noted as these are the times when early signs of disease and fresh ooze are likely (Billing, 1996). The likely time between a direct infection and the time when early signs of disease are seen is determined using degree-day sums above a mean temperature of 13 ºC, starting on the day after infection. Experience had shown that the best threshold sum for early signs of blight was ≥17. For early signs of blight, the DD12.7 ºC mean sum of 50 was only suitable for apple blossom blight (Billing, 2000). For blossom infection risks, BIS follows the principle that, when temperatures are high, flower wetting may be unnecessary (Mills, 1955; Billing, 1992).
Limitations. Limitations are related with other approaches such as the difficulty of obtaining adequate field records. Inoculum potential cannot be measured, only assessed broadly (Billing, 1996).

1.4.9. **Cougarblight.** It was developed for fire blight of pear and apple in Washington State (Smith, 1993). It uses temperature data to estimate the growth rate of fire blight bacteria over the past three days plus the present day, if wetting occurs in the afternoon or evening, or the previous four days if wetting occurs in the morning. Each day blossoms are open, the degree-hours for the noted days are added to obtain a four day degree-hour total. Degree-hour accumulation tables should be developed for each location of intended use based on regional weather station data. The goal is to determine what sort of growing conditions the bacteria have had while on the stigma during the approximately 96 hours prior to a wetting period. Two potential factors determine infection risk: potential climatic risk as described above, and one potential pathogen risk, related with fire blight history and fire blight in nearby orchards. Different versions exist according to year of development and temperature unit.

1.4.10. **Feuerbra** and **Anlafbra.** These are two programs developed in Germany for the assessment of regional and specific orchard risk. These two programs have been developed on the basis of the following three components: data on the weather risk, data on the monitoring pathogen and data on the orchard risk. These forecasting systems integrate parameters such as estimation of cultivar resistance or monitoring of the

pathogen in addition to weather data (Berger *et al.*, 1996). Some parameters are based on BRS. The IR days, were calculated following BRS method (Billing, 1992). For the estimation of D-period length, the equation includes daily PD values and rainfall. The orchard specific model, included data about phenological status of trees (such as secondary blossom and shoot succulence) and susceptibility details for every cultivar.

The more used warning systems are different versions obtained from those developed by Billing and Steiner. Although some of the fire blight warning systems rely on conclusions derived from laboratory experiments (e.g., effects of temperature on generation time of the bacterium), they all include rules that were developed without formal statistical analysis. Thus, they may all be considered qualitative empirical warning systems (Shtienberg *et al*., 2003).

As can be seen, all systems have advantages and disadvantages, but the most frequently used are those which have been updated and computerized, of easy use and interpretation, and are flexible. Among all available only BIS95 and Maryblyt fulfill these characteristics, and only Maryblyt is commercially available. German systems and Cougarblight also seem interesting but further evaluations need to be made. Finally Mills, BOS, and Parafeu at the moment are not very widely used as chemical treatment guide systems because they are obsolete, and need to be updated. BRS is used for elaborate phenoclimatological risk maps and the information generated by Powell's system can also be used for produce these maps (Montesinos and Llorente, 1999). The map of phenoclimatological risk aims to be a tool to locate geographically the prospections of symptoms and also to provide greater knowledge to the epidemiologist with the purpose of improving the effectiveness in the control of the disease.

1.5. Applicability of fire blight warning systems

The applicability of fire blight warning systems is limited because fire blight is a sporadic disease in space and time, which makes epidemiological studies and spray trials difficult to plan and the relative value of different approaches to risk assessment difficult to judge. The erratic occurrence of fire blight is attributed to differences in the availability of overwintering inoculum, alternative modes of spread of inoculum, the specific requirements governing infection, variations in specific local weather conditions, the stage of development of the cultivars available, alternative hosts, all of which makes the disease difficult and costly to control (Billing, 1984). In years of high disease incidence, there can be great differences between hosts, between cultivars and between locations. These differences largely depend on whether or not a host or a cultivar was in flower when weather-related risks were high. Such problems mean that statistical approaches to system and model design and their evaluation are rarely possible (Billing, 2000). Most commonly, high infection risk prediction is not followed by actual outbreaks in orchards. Less commonly, fire blight outbreaks occur when no infection was predicted (Smith, 1999a). Failure to detect low levels of disease is one of the greatest dangers. Blight does not always follow when the risks seem high. Blight may occur unexpectedly if a low incidence of disease has not been detected. One of the major factors limiting the improvement of present control programs has been our inability to predict the onset of fire blight epidemics accurately and reliably (Steiner, 1990).

1.6. Previous studies using fire blight forecast models in Spain

In Spain, fire blight was first detected in 1995, but has subsequently appeared in different outbreaks in Northeastern zone of the country. A preliminary study of risk prediction methods in the Northeast of Spain was performed by Montesinos and Llorente (1999) using Billing's revised system (BRS), Billing's integrated system (BIS95) and the Powell system modified to produce the weather risk maps. Results showed that the zones with high fire blight risk are the Ebro valley, and the zone between the valleys of the rivers Cinca and Gállego. A more detailed study clearly showed a region where the potential of fire blight is high around the Ebro river valley (between Huesca and Zaragoza) and Lleida (Figure 4.2). Results obtained with different models were very similar and showed that the potential of risk in this area is very high, in agreement with the fire blight foci detected in commercial orchards in the Northeast of Spain (Llorente *et al.,* 2002). This part of the work tries to continue the work initiated in our Plant Pathology Group by Montesinos and Llorente (1999).

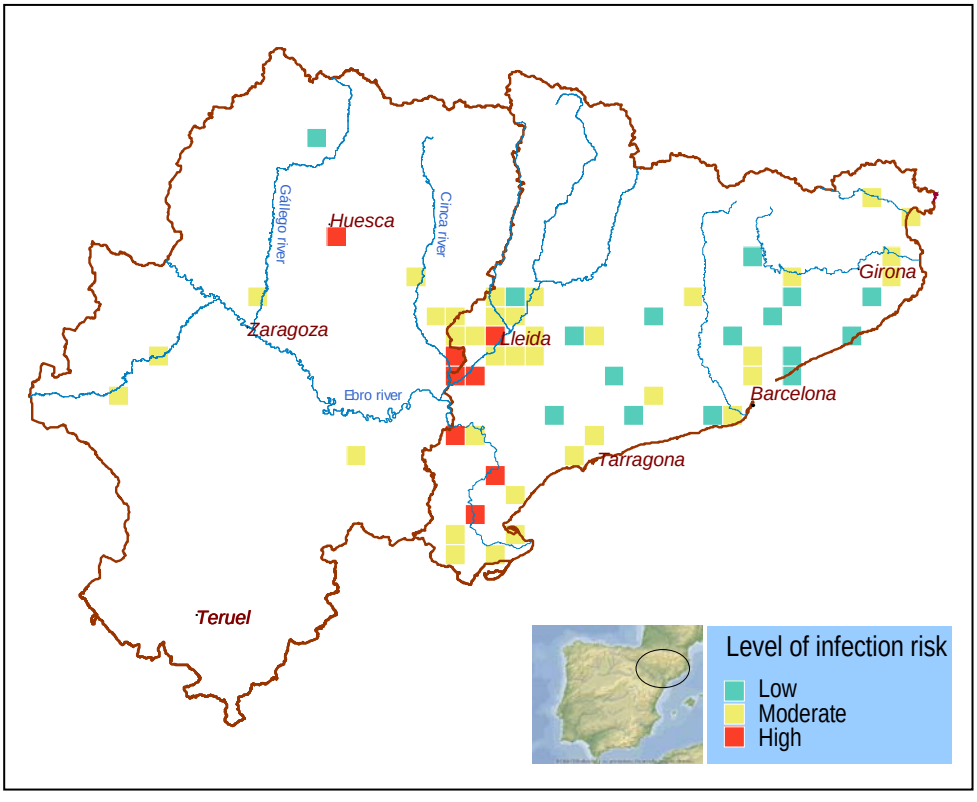


Figure 4.2. Map of fire blight risk in the Northeast of Spain according to modified BRS and Powell model, using historical weather data from a period of 5-10 years depending on weather station (from Llorente *et al.,* 2002).

OBJECTIVES

The main objectives of this work are:

1. To perform maps of fire blight risk based on historical weather data from recent years in Northeastern Spain to establish the regions where the probability of fire blight risk is high, to guide preventive inspections, and to determine the origin of outbreaks that had taken place.

2. To evaluate two models (Maryblyt, Cougarblight) for fire blight risk infection in an orchard naturally affected by fire blight in Spain in order to determine the accuracy of the predictions.

2. MATERIALS AND METHODS

2.1. MAPS OF FIRE BLIGHT RISK ACCORDING TO WEATHER DATA IN NORTHEASTERN SPAIN

In this work we have examined several fire blight warning systems that had been developed for fire blight management in other locations, with the aim of determining in space and time the fire blight infection risk. Historical weather data were used. The analysis was made in Northeastern Spain (Aragón and Catalunya).

2.1.1. Weather data

Data were collected from 69 locations in Northeastern Spain over three years (2000, 2001 and 2002) (Figure 4.3). Weather variables used were; daily maximum temperature (ºC), minimum temperature (ºC) and precipitation (mm). The weather stations belonged to the regional network of Xarxa Agrometeorològica de Catalunya (XAC) from the Departament Agricultura Ramaderia i Pesca (Generalitat de Catalunya) and to Red Regional de Estaciones Meteorológicas de Aragón (Instituto Nacional de Meteorología). The network has 58 weather stations distributed all over the region in Catalunya, and 11 weather stations from Aragón.

2.1.2. Weather data collection

Daily weather data were recorded by remote weather stations and dataloggers (CR10, CR10X or CR500, Campbell Scientific Ltd., UK) connected to sensors (Campbell Scientific Ltd., UK) for temperature and rainfall measuring. Data from Catalunya were real time available on the XAC website, and were transferred via internet. Data from Aragón were transferred from the Centro de Protección Vegetal (Gobierno de Aragón).

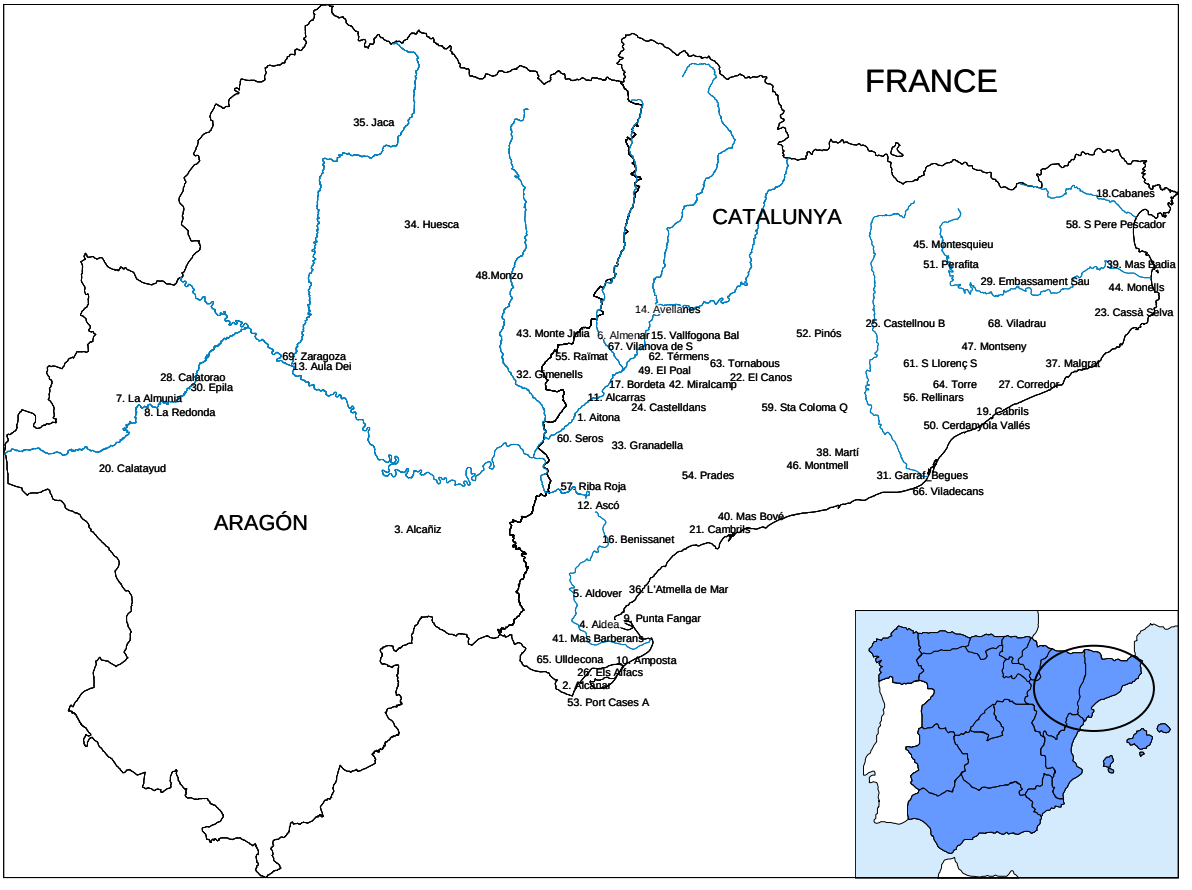


Figure 4.3. Map of geographical location of the weather stations used in the elaboration of fire blight risk maps.

2.1.3. Fire blight risk models used

Systems used are: BRS, Powell, BIS95 and Feuerbra (previously described in pp. 130,131 and 132). Models were transformed into a computer program using dBase IV software (Version 1.0, Borland International Inc., CA, USA) and modified to simplify their use. The weather data recorded (maximum and minimum daily temperature and rainfall) and phenology corresponded to the input data, calculated data performed by models were the values which were used for predicted output events (date of start of infection, length of infection period or date of first visible symptom). Phenology was estimated, and months of March-May were considered as potential period favorable to infection.

Billing's revised system (BRS). In BRS fire blight prediction system, the calculated values are used to estimate the start of infections and the duration of incubation period until the appearance of first visible symptom. An incubation period is expected to be completed when the number of cumulative potential doublings (after infection) exceeds a certain threshold. The BRS calculations performed were as described below:

1. Potential doubling (PD) was calculated from maximum and minimum temperature, as described by Schouten (1987) (Table 4.2).

Table 4.2. Estimated potential doublings per day (PD) in relation to daily minimum and maximum temperatures (Tmin and Tmax) (Schouten, 1987)

Tmax (ºC)	Tmin (ºC)																		
	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
0	0.0																		
2	0.0																		
4	0.1	0.2	0.3																
6	0.3	0.4	0.5	0.7															
8	0.6	0.7	0.9	1.1	1.4														
10	1.0	1.1	1.4	1.6	2.0	2.4													
12	1.6	1.7	2.0	2.3	2.7	3.2	3.8												
14	2.3	2.5	2.8	3.2	3.6	4.2	4.8	5.5											
16	3.1	3.4	3.7	4.2	4.7	5.3	5.9	6.7	7.6										
18	4.1	4.5	4.8	5.3	5.9	6.5	7.2	8.0	9.0	9.9									
20	5.3	5.6	6.1	6.6	7.2	7.8	8.6	9.5	10.4	11.4	12.5								
22	6.5	6.9	7.3	7.9	8.5	9.3	10.1	11.0	11.9	12.9	14.0	15.0							
24	7.7	8.1	8.7	9.2	9.9	10.7	11.5	12.4	13.4	14.4	15.4	16.5	17.4						
26	8.9	9.3	9.9	10.5	11.2	12.0	12.8	13.7	14.7	15.7	16.7	17.7	18.5	19.2					
28	9.9	10.4	10.9	11.5	12.2	13.0	13.9	14.8	15.7	16.7	17.6	18.5	19.2	19.7	20.0				
30	10.6	11.1	11.6	12.2	12.9	13.7	14.5	15.4	16.3	17.1	18.0	18.7	19.3	19.7	19.7	19.3			
32	10.8	11.3	11.8	12.4	13.1	13.8	14.6	15.4	16.2	17.0	17.7	18.3	18.7	18.9	18.7	18.0	16.8		
34	10.5	10.9	11.4	12.0	12.6	13.3	14.0	14.7	15.4	16.0	16.6	17.0	17.2	17.1	16.7	15.7	14.2	12.2	
36	9.5	9.9	10.4	10.9	11.4	12.0	12.6	13.2	13.8	14.3	14.6	14.9	14.8	14.5	13.7	12.5	10.7	8.4	5.5

2. A rain score (R) is assigned as:
   R = 1 if rainfall ≥ 2.5 mm
   R = 0.5 if rainfall ≤ 2.4 mm and ≥ 0.1 mm
   R = 0.5 if rainfall = 0 mm and PD ≥ 11
   R = 0 if rainfall = 0 mm and PD < 11

3. The infection risk score (IRS) is calculated from PD and R values as described by Billing (Table 4.3). The IRS ranges from 1 to 7.

Table 4.3. Infection risk score (IRS) method (from Billing, 1990)

PD on day of rain or on day before	IRS Score when daily rainfall (mm) ≥					
	0	**<0.1**	**1.0**	**2.5**	**10**	**20**
<5				1	1	1
5.0-6.9			1	2	3	4
7.0-8.9			1	3	4	5
9.0-10.9		1	2	4	5	6
≥ 11	2[a]	2	3	5	6	7

[a] Score is 2 only during bloom period

4. Prediction of the start of infection time is achieved by means of IRS score, and according to BRS the infection begins when IRS score is greater or equal to 2.

5. In order to predict when first visible symptom appears the length of the disease development period (D) was calculated. Calculations of D-period begin on days when the IRS score is greater or equal to 2. This is considered as day zero. The disease development period ends when $\sum PD \geq (36t/\sum R)-6$. Where $\sum PD$ is the sum of the PD score since day zero, t is the number of days, and $\sum R$ is the cumulative rain score since day zero. The minimum $\sum PD$ for D-period completion is 30, and the minimum days (t) for completion are 3. The equation shows that when the weather is wet, less heat is needed to complete the incubation period, and when the weather is dry, more heat is needed to complete the incubation period.

Powell. The Powell's system used temperature criteria for prediction of apple and pear blossom blight. There are different methods that attempt to calculate the heat accumulation above a minimum threshold temperature; in our work the following was used;

1. With maximum and minimum temperature the degree-days (DD) were calculated according to the single triangulation method, using 18.3 ºC as lower threshold and 36 ºC as upper, as described by Powell (1965). The degree-days were calculated from 1 January to 31 May of each year.
2. The sum of degree-day above 18.3 ºC was calculated.
3. The start of risk period was noted as the date when the sum of degree-day was above 18.3 ºC.

Billing's integrated system, 1995 (BIS95) modified. Partial information generated from BIS95 (Billing, 1996) was used. Original BIS95 allows prediction of the blossom blight risks and the time when early signs of disease and fresh ooze are likely. In our work we only used the data that calculate the blossom blight risk.

1. The degree-days (DD) were calculated from maximum temperature as daily values of 1 ºC or more above 18 ºC and are counted as DD.
2. The sum of degree-day above 18 ºC (DD18 max) was calculated from 1 April to 31 May.
3. The blossom blight risk was noted as the date when the sum of DD18 max was above 17 or multiples of 17 (34, 51, 68, 85,…).

Feuerbra. According to the Feuerbra forecasting program, fire blight risk can be adjusted depending on disease resistance of the apple or pear variety (Berger *et al*., 1996). In this work the risk level was calculated following the original model, as described below.

1. Potential doubling (PD) was calculated from maximum and minimum temperature as described by Schouten (1987).
2. A rainfall score (F) was assigned as:
   F = 0.32 if rainfall > 2.5 mm
   F = 0.26 if rainfall ≤ 2.5 mm and rainfall > 0 mm
   F = 0.24 if rainfall = 0 mm
3. PD values were multiplied by F.
4. Start of incubation period is identical with BRS, infection risk score (ISR) is multiplied by factor 10.

5. Four different groups of cultivars were established according to their susceptibility to fire blight (Table 4.4).

Table 4.4. K-factor according to fire blight susceptibility of cultivar (Berger *et al.*, 1996)

Group cultivar	k-factor
High susceptibility	1
Susceptible	0.8
Low susceptibility	0.4
Resistant	0.1

6. End of incubation period corresponds to the appearance of first symptoms, and first visible symptoms appear when k∑PD*F > 29.89; where k is a factor related to the susceptibility, PD is the potential doubling, F is rainfall factor.

Table 4.5 summarizes the input parameters used, the calculations performed and the information generated by the models.

Table 4.5. Input, calculated and output data of each fire blight forecast model tested

Model	Input			Calculations[a]				Output	
BRS	Tmax	Tmin	Rain	PD	R	IRS		Start of infection (IRS ≥ 2)	First symptom D-period ∑PD ≥ (36t/∑R)-6
Powell	Tmax	Tmin		DD				Start of risk period (∑DD ≥ 18.3 ºC)	
BIS95 modified	Tmax			DD18				Blossom blight risk (∑DD18 ≥ 17 or multiples of 17)	
Feuerbra	Tmax	Tmin	Rain	PD	F	IRS	k	Start of infection (IRS ≥ 2)	First symptom D-period (k∑PD*F > 29.89)

[a] PD: potential doubling; R: rain score; IRS: infection risk score; DD: degree days; F: rain score; k: fire blight susceptibility cultivar

2.1.4. Data analysis

A new risk was obtained for each weather station location and year, with calculated values and output data obtained from different models. Risk level obtained was located on a map.

Risk obtained from combination of BRS and Powell. Partial information generated from BRS (Billing, 1990) and Powell (1965) was used. New disease index risks were established according to the number of potential days of infection (n) from March to May, the mean duration of predicted infection periods (d), and date account of cumulated 18.3 degree-day above a threshold of 18.3 ºC according to previous studies (Montesinos and Llorente, 1999; Llorente *et al.,* 2002).

1. For each weather station and year the **number** of D-periods from March to May was also calculated. According to the number of D-period a new index was calculated **In** (Table 4.6). The threshold level was obtained using an analysis of frequencies using data of 10 years (1990-1999) collected from 72 weather stations. The relationship between the number of D-periods and the infection risk was directly related.

2. For each weather station and year the **mean** of D-period from March to May was calculated. Long D-period has a reduced infection risk therefore D-periods higher than 20 days were excluded. On the basis of the mean of D-period a new index was calculated **Id**. The level threshold was calculated as described above, using data from 10 years (Table 4.6). The mean of D-period and infection risk were inversely related (when D-period is high, Id is low).

3. The day in which the sum of degree-day is above 18.3 ºC is related with infections as described in Powell model. The date account of cumulated 18.3 ºC degree-day above a threshold of 18.3 ºC was used to obtain a new index **It** (Table 4.6).

Table 4.6. Partial risk **In**, **Id**, and **It** according to the length of infection period, number of infection risk periods, and date above 18.3 DD, respectively (modified from Montesinos and Llorente, 1999)

Number of infection risk (n)	Risk In	Length of infection period (days)	Risk Id	Data above DD≥18.3 ºC	Risk It
n < 20	1	d < 4	3	1-20 April	3
20 ≤ n < 32	2	4 ≤ d ≤ 5	2	21 April – 10 May	2
n ≥ 32	3	d > 5	1	11-31 May	1

Overall risk depends on partial risk of mean of length of infection period, number of infection risk, and data where sum of degree days is above the threshold 18.3 ºC (Table 4.7). The risk level was calculated for each year and weather station as follows, overall risk = **In · Id · It**.

Table 4.7. Risk level according to overall risk index (Montesinos and Llorente, 1999; Llorente *et al.,* 2002)

Overall Risk	Risk level
(In*Id*It) ≤ 4	**low**
4 < (In*Id*It) < 12	**moderate**
(In*Id*It) ≥ 12	**high**

Risk according to modified BIS95. For each year and weather station the number of times that the sum of degree-days reaches 17 or multiple of 17 (s) was considered a start of potential infection. A new index (**Is**) was generated: 1 for s < 5; 2 for 5 ≤ s ≤ 11 and 3 for s > 11 (Table 4.8).

Table 4.8. Risk level of fire blight infection according to modified BIS95 model (Llorente *et al*., 2002)

Number of dates when the sums reach 17 (s)	Risk Is	Risk level
s < 5	1	**low**
5 ≤ s ≤ 11	2	**moderate**
s > 11	3	**high**

Risk according to Feuerbra. For each year and weather station four types of infection periods were obtained according to host susceptibility as previously described. An analysis of frequencies was performed with the **mean** of D-period calculated for each weather station.

Weather-based fire blight risk maps. Weather-based fire blight risk maps of the Northeast of Spain (Catalunya and Aragón areas) were elaborated by a Geographic information system (GIS). A GIS is a computer system capable of assembling, storing, manipulating, and displaying data referenced by geographic coordinates (Star and Estes, 1990). In this work we used GIS to improve the spatial accuracy of weather information to assist in the real-time predictive fire blight models. Maps were generated with MiraMon® software (Version 3.5i, CREAF, Catalunya, Spain). Maps were elaborated with data from 3 years (2000, 2001 and 2002) and 69 weather stations using information of Billing's revised system (BRS) combined with Powell and Billing's integrated system, 1995 (BIS95).

2.2. Predicting fire blight infections using the Maryblyt and Cougarblight models in a commercial orchard in Northeastern Spain

A preliminary evaluation of two available models (Maryblyt and Cougarblight) was performed in a commercial orchard in Northeastern Spain (Zaragoza), affected by fire blight in 2001.

2.2.1. Orchard and weather observations

Detailed observations on pear phenology, daily and cumulative weather factors and fire blight development were made in a blight prone area of a commercial orchard in Zaragoza (Las Llanas, Epila) in 2002. The orchard area was 25 ha with 29,869 trees, and divided into 24 sectors (41º 36' 14.93'' N; 4º 39' 50.88'' E) (Figure 4.4). Pear cultivars were mainly Williams and some sectors contained Blanquilla and Conference, and some rows corresponded to Doyenne du Comice, 2% of Red Crimson and 0.2% of Abate Fetel. Pear trees were monitored twice per week from green tip to

petal fall. Visual prospections were carried out by Centro de Protección Vegetal de la Diputación General de Aragón. A weather station was installed in the middle of the study area. Environmental variables were monitored with a datalogger (CR10X, Campbell Scientific Ltd., UK) connected to combined temperature-relative humidity (HMP35AC, Vaisala, Finland), wetness (237, Campbell Scientific), and rainfall (ARG 100, Campbell Scientific) sensors. Temperature and RH were measured at 10 min intervals and wetness and rainfall every 20 s and both measurements were recorded by the datalogger at hourly intervals from 8:00 to 8:00 h (GMT).

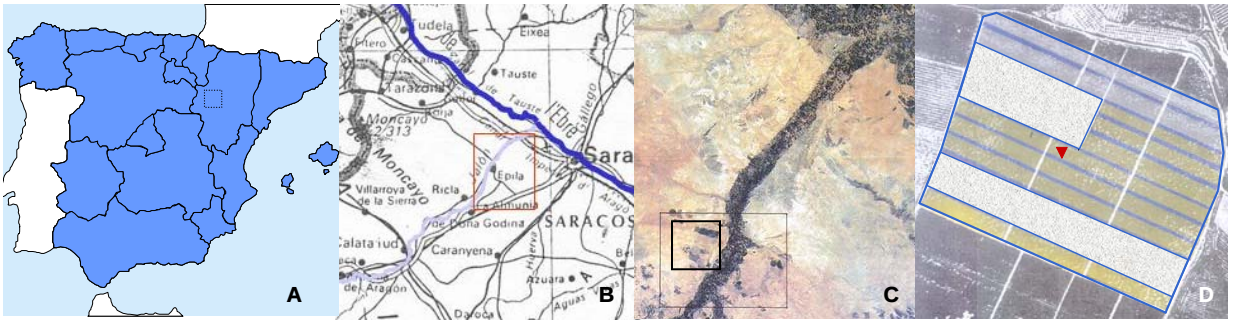


Figure 4.4. Location of orchard Las Llanas. Spain map (A), Zaragoza map with Ebro and Jalón rivers and orchard of study (red rectangle) (B), aerial photograph of Jalón river (C), and aerial photograph of orchard of study with sectors, indicating the location of weather station (red triangle) and area affected by fire blight the previous year (2001) (white rectangles) (D) (modified from M.A. Cambra).

2.2.2. Phenology and bloom records

The phenological stage of trees was determined at 2-3 day intervals from dormant buds through the completion of petal fall in 2001 and 2002. The determination was performed following the Fleckinger phenological stage description, and then a correspondence with bud development stage described and used in Maryblyt model was established (Table 4.9).

Table 4.9. Phenological stage described by Fleckinger (1965) and correspondence with bud development stage used by Maryblyt model

Fleckinger stage	Description	Phenological stage used by Maryblyt
A B	Dormant and slightly swollen buds	Dormant or silver tip (ST)
C C_3 D D_3	Swelling buds appearance of the floral buds	Green tip (PG)
E E_2 F	The sepals let the petals appear first flower	First bloom (B)
F_2 G	Full bloom and fall of the first petals	Some secondary bloom remains (B_2)
H	Fall of last petals	Petal fall (PF)

2.2.3. Description of the warning systems used

Two models were used for predicting fire blight infection risk: Maryblyt and Cougarblight.

Maryblyt model computes daily maximum and minimum temperature, rainfall or dew events and tree phenological stage to predict blossom, canker, shoot, and trauma blight. The model integrates the use of three cumulative heat units to indirectly monitor the development of host, pathogen population, insect vector availability and symptom development (Steiner, 1990). To calculate degree-days (DD) and degree-hours (DH) the program uses a sine wave function with a maximum temperature of 32ºC, and various minimum temperature thresholds.

1. The cumulative DD>4.4 ºC monitors the age of flowers and the appearance of insect vectors.
2. The cumulative DH>18.3 ºC is used to establish epiphytic infection potential (EIP), which is an index for assessing infection risk. EIP is calculated by expressing the number of DH>18.3 ºC accumulated over the last 44.4 or 66.7 DD>4.4 ºC for apples or pears, respectively, as a percentage of 110 DH>18.3 ºC, which represents the threshold for infection. EIP is based on the assumption that abundant inoculum is available in and around the orchard.
3. The cumulative DD>12.7 ºC, is used to predict symptom development, once infection has occurred.

The program calculates mean temperature, epiphytic infection potential (EIP), and indicates if the blossoms are open (B), if EIP≥100 (H), if there is a wetting event (W) and if average daily temperature≥15.6ºC (T). Integrating all these data the model displays a simple plus (+) or minus (-) BHWTR method which indicates the minimum requirements needed to incite a blossom blight epidemic, where R is the risk for infection. According to the model, risk is **low** if one of the four conditions for blossom infection is met, **moderate** when there are two conditions and **high** when there are three conditions, an **infection event** (I) is signaled when all four blossom infection factors occur. When all of these minimum conditions are met in sequence, infection occurs and first **blossom blight** symptoms are expected after an accumulation of 57 DD>12.7 ºC from infection date. **Canker blight** symptom predictions occur in two phases. First phase corresponds to canker margin symptoms (CMS) as a percentage of the minimum threshold for this event (109 DD>12.7 ºC after green tip), when the CMS threshold is met, second phase starts with more visible canker blight symptoms (166 DD>4.4 ºC after green tip). **Shoot blight** symptom prediction as a percentage of a variable minimum threshold determined by the time that either BBS or CBS appear and when the insect vector threshold (default for white apple leafhopper adults = 375 DD>4.4 ºC after green tip) is met. **Trauma blight** symptom predictions are triggered when over 110 DH>18.3 ºC accumulate and severe trauma events such as late frost (-2.2 ºC), hail storms or high winds damage the foliage.

Cougarblight model uses daily maximum and minimum temperature, rainfall and leaf wetness as weather variables, and the potential for pathogen presence due to proximity of fire blight cankers, to evaluate the potential risk of infection. According to Cougarblight 98C (Smith, 1999a), the relative risk of fire blight is calculated each day that blossoms are open in the orchard. This model should be used from when the orchard enters bloom until only a few blossoms remain. Risk is dependent on the presence or past presence of fire blight and leftover cankers. An adaptation of original model was used, and only in the wetting days the degree hour were calculated.

1. From hourly average temperature and using daily degree-hour estimation chart (Table 4.10) the sum of degree hours over the past 4 consecutive days is calculated, each day those blossoms are open.
2. The presence or past presence of fire blight in the orchard is determined as described in the table below (Table 4.11). In our study we use the potential for pathogen presence as fire blight in the orchard or the neighbor orchard last year (2001).
3. To determine the risk prediction level, the 4 day cumulated degree-hour that exceeded the threshold of infection relative risk according to the presence of fire blight is rated as either low, moderate, high, or extreme.

Table 4.10. Daily degree-hour (DDH) estimation chart (Smith, 1999b)

Temp[a]	DDH	Temp	DDH
15	0	29	15.4
16	0.3	30	15.5
17	0.9	31	15.4
18	1.7	32	14.9
19	2.6	33	13.9
20	3.7	34	12.6
21	5.1	35	10.6
22	6.7	36	7.8
23	8.4	37	4.8
24	10.5	38	2.5
25	12.3	39	1
26	13.9	40	0.3
27	14.8	41	0
28	15.2		

[a] Temp: average temperature during 1 hour

Table 4.11. Infection risk relative to the 4 day cumulated degree-hour for potential pathogen presence (Smith, 1999b)

Potential for pathogen presence	Low	Caution	High	Extreme
No fire blight in the area for the past season	0-200	200-270	270-430	430+
Fire blight in the orchard or the neighbor orchard last year	0-110	110-160	160-270	270+
Active fire blight cankers are now in or very near the orchard	0-30	30-110	110-200	200+

3. RESULTS

3.1. MAPS OF FIRE BLIGHT RISK ACCORDING TO WEATHER DATA IN NORTHEASTERN SPAIN

Maps of fire blight risk were made over the three years of study with overall risk obtained from combined Billing's revised system-Powell (BRS-Powell) model and from modified Billing's integrated system, 1995 (BIS95) for each weather station location. Risk obtained from combined BRS-Powell model showed lower risk than obtained from modified BIS95 in the three years of study (Table 4.12). In 2000 the number of locations with low infection risk according to BRS-Powell model was 23, while according to modified BIS95 model was 4. In 2002, differences increased and the number of locations with low risk according BRS-Powell model was 40 and according to modified BIS95 was 6. The trend for the risk over time was, to diminish from 2000 to 2002, above all in the low risk zone. Table 4.13 summarizes the potential fire blight risk according to the two models for 69 weather stations. Results obtained by the two models were different, although both showed that the potential of risk in the area around the Ebro river valley (between Huesca and Zaragoza) and Lleida, was very high, and in the area including Girona and Barcelona the disease risk was lower, showing two clearly differentiated regions according to the potential risk of fire blight (Figures 4.5 and 4.6).

Table 4.12. Number of weather stations for each fire blight risk level according to combined Billing's revised system-Powell model (BRS-Powell), and Billing's integrated system, 1995 (BIS95) for 2000, 2001 and 2002

	Model					
	BRS-Powell			BIS95		
Year	Low	Moderate	High	Low	Moderate	High
2000	23	12	34	4	22	42
2001	27	25	17	2	17	49
2002	40	11	15	6	33	28

Table 4.13. Fire blight risk obtained according to combined Billing's revised system-Powell model (BRS-Powell), and Billing's integrated system, 1995 (BIS95) in each weather station for 2000, 2001 and 2002

		Year					
		2000		2001		2002	
Code	Station	BRS-Powell[a]	BIS95	BRS-Powell	BIS95	BRS-Powell	BIS95
1	AITON	3	3	3	3	3	3
2	ALCAN	2	2	2	2	1	2
3	ALCAÑ	3	3	3	3	2	3
4	ALDEA	3	3	2	3	3	3
5	ALDOV	3	3	2	3	1	3
6	ALMEN	3	3	2	3	1	3
7	ALMUN	3	3	3	3	3	3
8	ALRED	1	3	3	3	2	3
9	AMPOL	3	2	2	3	3	2
10	AMPOS	2	2	2	3	2	2
11	ARRAS	3	3	2	3	3	3
12	ASCOR	3	3	3	3	1	2
13	AULAD	3	3	3	3	1	3
14	AVELL	1	3	1	3	1	2
15	BALAG	3	3	2	3	2	3
16	BENIS	3	3	2	3	3	3
17	BORDT	3	3	3	3	3	3
18	CABAN	3	3	1	3	3	2
19	CABRI	1	2	2	2	1	1
20	CALAT	2	2	1	2	1	2
21	CAMBR	2	2	2	2	1	2
22	CANOS	2	3	1	3	1	2
23	CASSA	1	2	1	3	1	2
24	CASTE	3	3	3	3	3	3
25	CBAGE	2	2	1	3	1	2
26	CNAQU	3	3	2	2	2	2
27	CORRE	1	2	1	2	1	1
28	CTORA	2	nd	2	nd	nd	nd
29	EMSAU	3	3	1	3	1	2
30	EPILA	2	3	3	3	2	3
31	GARRA	1	1	1	2	1	1
32	GIMEN	3	3	2	3	2	3
33	GRANA	1	3	2	3	1	2
34	HUESC	1	2	1	3	1	3
35	JACA	1	2	1	2	1	2
36	LAMET	1	2	3	3	2	2
37	MALGR	1	2	1	2	1	2
38	MARTI	2	3	2	3	1	3
39	MASBA	3	3	1	3	2	2
40	MASBO	3	3	2	3	1	2
41	MBARB	1	3	2	3	3	2
42	MCAMP	3	3	2	3	1	3
43	MJULI	3	3	3	3	3	3
44	MONEL	1	3	2	3	1	2
45	MONTE	1	2	1	3	1	2
46	MONTM	3	2	1	2	1	2
47	MONTS	1	1	1	1	1	1
48	MONZO	3	3	3	3	3	3
49	PANGL	3	3	2	3	1	3
50	PARCT	2	3	3	3	1	2
51	PERAF	1	2	1	2	1	2
52	PINOS	1	2	1	2	1	2
53	PORTC	2	2	1	2	nd	nd
54	PRADE	1	1	1	1	nd	1
55	RAIMA	2	3	1	3	1	3
56	RELLI	3	3	3	3	1	2
57	RIBAR	3	3	2	3	3	3
58	SANPE	1	2	1	2	1	2
59	SCQUE	1	2	1	2	1	2
60	SEROS	3	3	2	3	3	3
61	SLLSA	1	2	1	3	1	2
62	TERME	3	3	3	3	2	3
63	TORNA	3	3	1	3	2	3
64	TORRE	3	3	3	3	1	2
65	ULLDE	3	3	2	3	1	2
66	VILAD	1	2	1	2	1	2
67	VISEG	3	3	2	3	1	3
68	VLADR	1	1	1	2	1	1
69	ZARAG	3	3	3	3	3	3

[a] overall risk; 1: low; 2: moderate and 3: high; nd: not determined

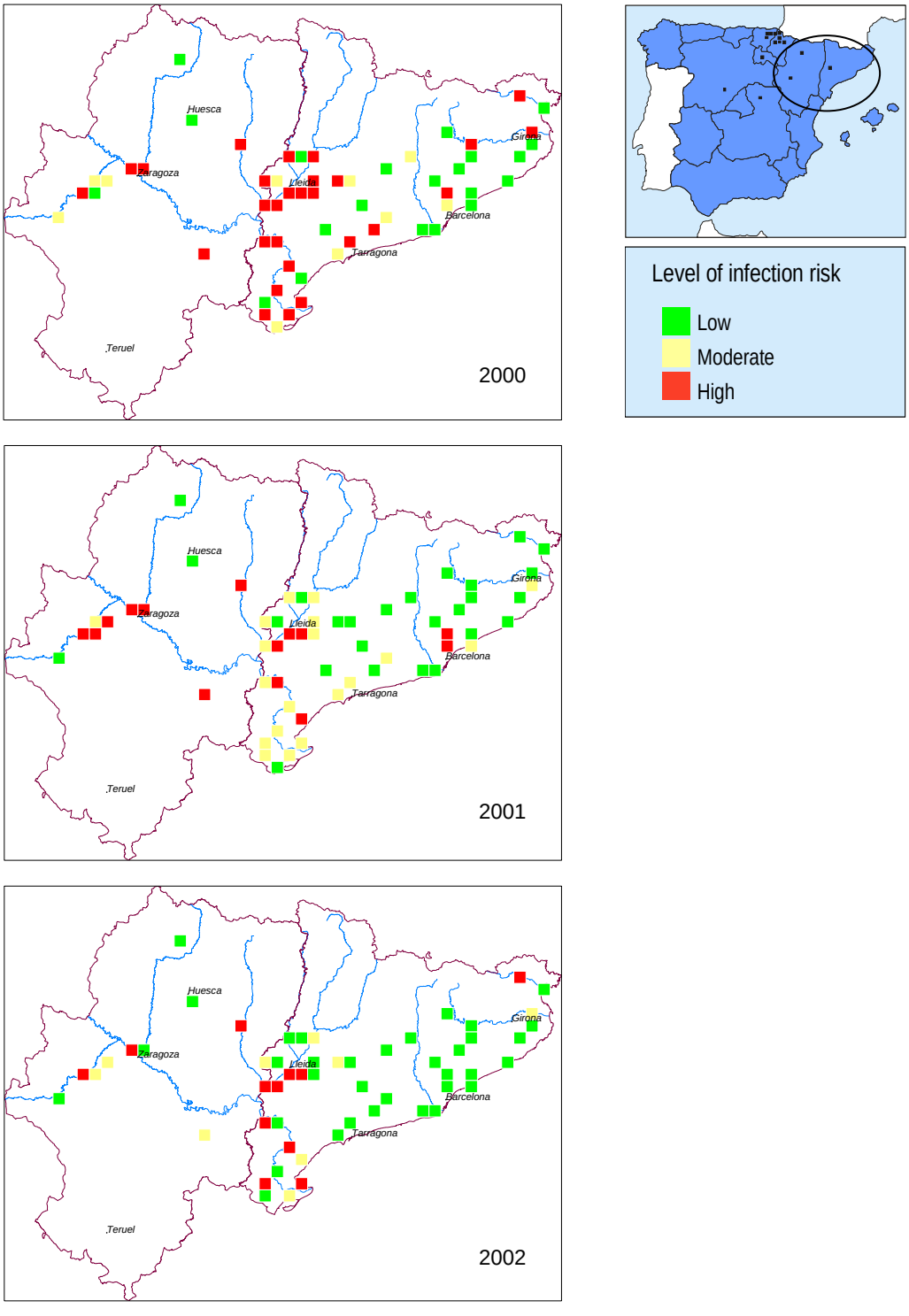


Figure 4.5. Maps of fire blight risk in the Northeast of Spain obtained according to combined Billing's revised system and Powell model (BRS-Powell), for 2000, 2001 and 2002. Black points in Spain map on the right represent fire blight outbreaks described since 1995 to 2002.

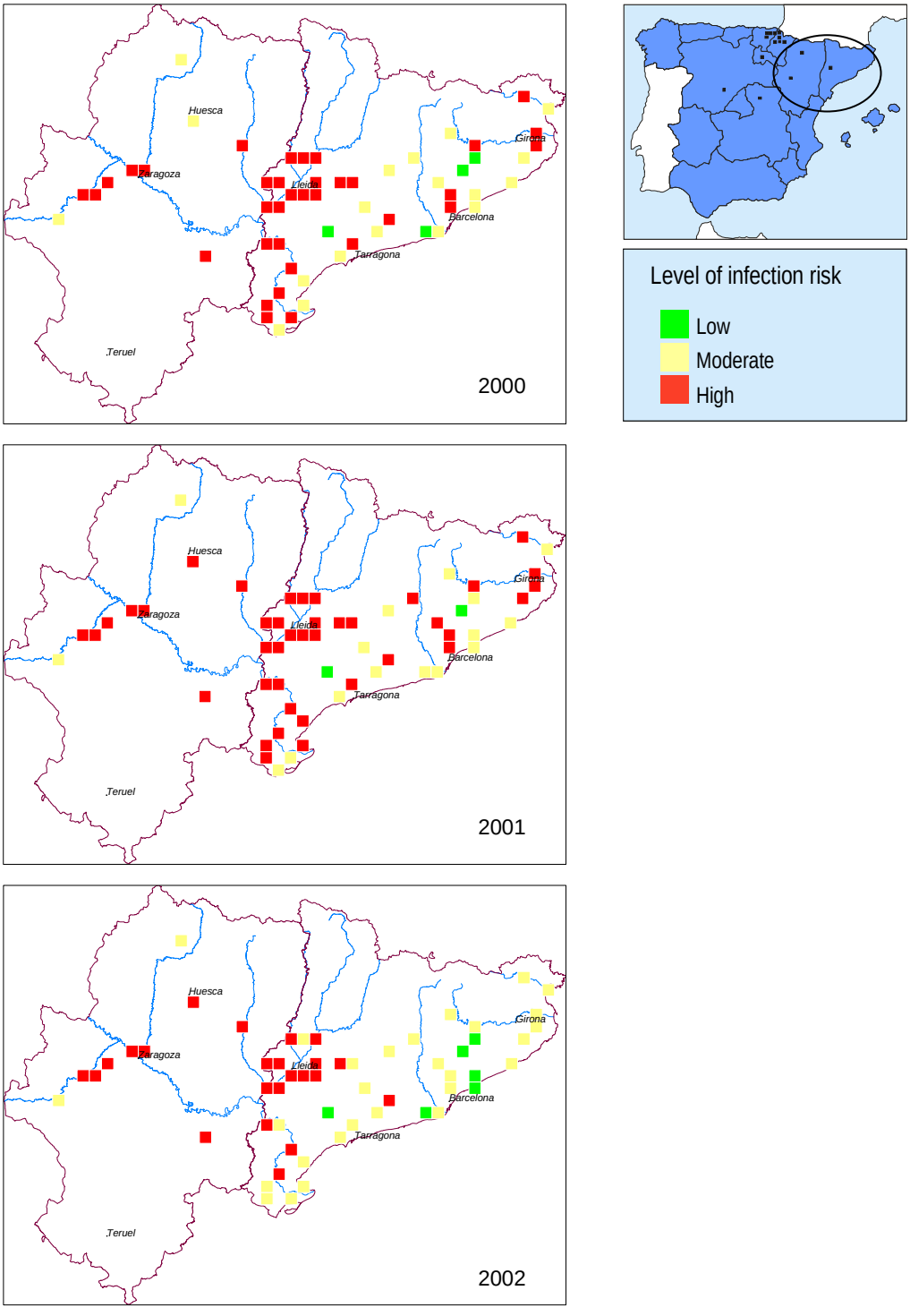


Figure 4.6. Maps of fire blight risk in the Northeast of Spain obtained according to modified Billing's integrated system, 1995 (BIS95) in each weather station for 2000, 2001 and 2002. Black points in Spain map on the right represent fire blight outbreaks described since 1995 to 2002.

Length of infection periods obtained with Feuerbra model from 69 locations of three years of study were compared to periods obtained with BRS, a box diagram was performed (Figure 4.7). According to Feuerbra model the length of infection period varies depending on the host susceptibility. An increased susceptibility reduces the length of infection period. Between low susceptible host and moderately susceptible differences in the length of d-periods were lower, while with low susceptible or resistant hosts the length of infection period increased markedly. In comparison with data obtained from Billing's revised system, for all types of host the length of infection period was higher than those from Feuerbra model.

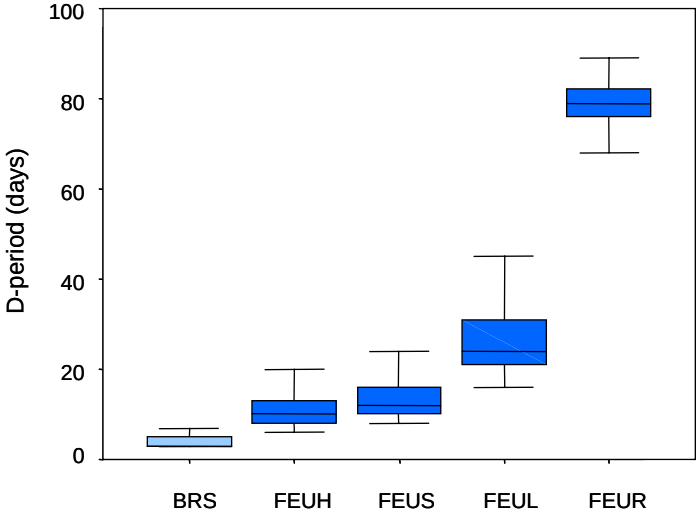


Figure 4.7. Box plot of length of infection period obtained with Feuerbra model for each level of host susceptibility: high (FEUH), susceptible (FEUS), low (FEUL) and resistant (FEUR) in comparison with Billing's revised system (BRS). Analysis was performed with data from 69 locations of three years (2000, 2001 and 2002).

3.2. Predicting fire blight infections using the Maryblyt and Cougarblight models in a commercial orchard in Northeast of Spain

Maryblyt model predicts the fire blight infection risk and the appearance of blossom, canker, shoot and trauma blight symptom for the two pear cultivars Blanquilla and Williams (Figure 4.8) from weather and phenological data recorded (Table 4.14). Between the two cultivars tested slight differences were observed related to the date of first bloom. As date of first bloom for Williams was later than for Blanquilla the fire light infection risk periods were different. In reference to the appearance of blight symptoms there were also some differences related to the initial date of first symptom, but not to the number or progression. Trauma blight periods were observed at first and

end July. According to Maryblyt model two infection periods were predicted for both cultivars on 23 May and from 1 to 5 June that go one until the end of blossom period (5 June). Consequently six blossom blight symptoms were predicted on 1, 7, 8, 10, 12 and 13 June in both cultivars (Table 4.15). The model also predicted the apparition of two canker blight symptoms on 8 and 25 April for cv. Blanquilla and on 17 and 26 April for cv. Williams. In 13 May a shoot blight symptom was predicted in both cultivars as consequence of inoculum released by canker blight and insect activity. Finally three trauma blight symptoms were predicted, on 23 May as consequence of infection by frost on 5 April, on 16 July and on 2 August as consequence of two infections initiated by hail on 8 and 27 July, respectively. According to Cougarblight model four infection risk periods were predicted, the first three periods agreed with the high infection risk periods predicted by Maryblyt model, and the last infection period predicted on 3 June by Cougarblight is in agreement with the second infection period predicted by Maryblyt (Figure 4.9). Wetting days from Cougarblight model must be considered as high risk days, in spite of the risk level obtained by accumulated degree-hour was low. The data were adjusted to blossom period considering the blossom period until 5 June, although at the end May and first June, only some secondary bloom remains in the orchard, with lower amount of flowers (Table 4.14). However, although both model predicted infection risk periods, no fire blight symptoms were detected in field prospections.

Table 4.14. Date of phenological stage of pear cv. Blanquilla and Williams in Las Llanas, in 2002

Maryblyt phenological bud stage	Blanquilla	Williams
Dormant or silver tip (ST)	20/02	20/02
Green tip (PG)	25/02	04/03
First bloom (B)	11/03	18/03
Some secondary bloom remains(B_2)	31/03	31/03
Petal fall (PF)	06/06	06/06

Table 4.15. Number and date of predicted infection risk, blossom blight, canker blight, shoot blight and trauma blight symptoms by Maryblyt in pear cv. Blanquilla and Williams in Las Llanas, in 2002

	Infection		BBS[a]		CBS		SBS		TBS	
Number	B[b]	W	B	W	B	W	B	W	B	W
1	23/05	23/05	01/06	01/06	08/04	17/04	13/05	13/05	23/04	23/04
2	01/06	01/06	07/06	07/06	25/04	26/04			16/07	16/07
3	02/06	02/06	08/06	08/06					02/08	02/08
4	03/06	03/06	10/06	10/06						
5	04/06	04/06	12/06	12/06						
6	05/06	05/06	13/06	13/06						

[a] BBS: blossom blight symptom; CBS: canker blight symptom; SBS: shoot blight symptom; and TBS: trauma blight symptom
[b] B: Blanquilla; W: Williams

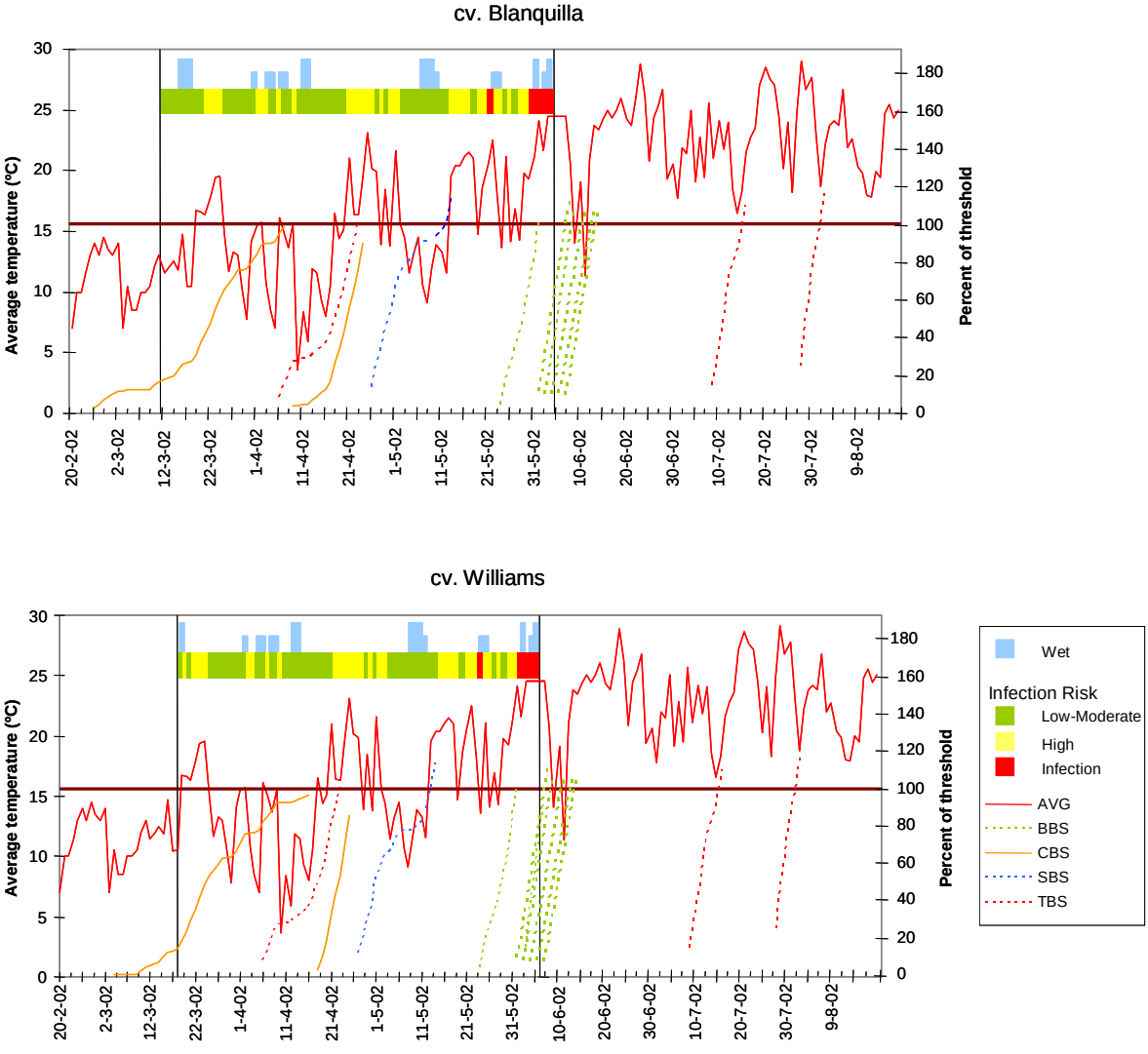


Figure 4.8. Fire blight infection risk obtained by Maryblyt model for two pear cultivars Blanquilla and Williams, in Las Llanas in 2002. Horizontal bars represent the wetness periods, and predicted infection risk. Red line is the average temperature (AVG). The dashed green line represents the progression of blossom blight symptom as percentage (BBS); orange line plots the canker blight progression (CBS), dashed blue line represents the shoot blight symptom (SBS) and dashed red line plots the trauma blight progression (TBS).

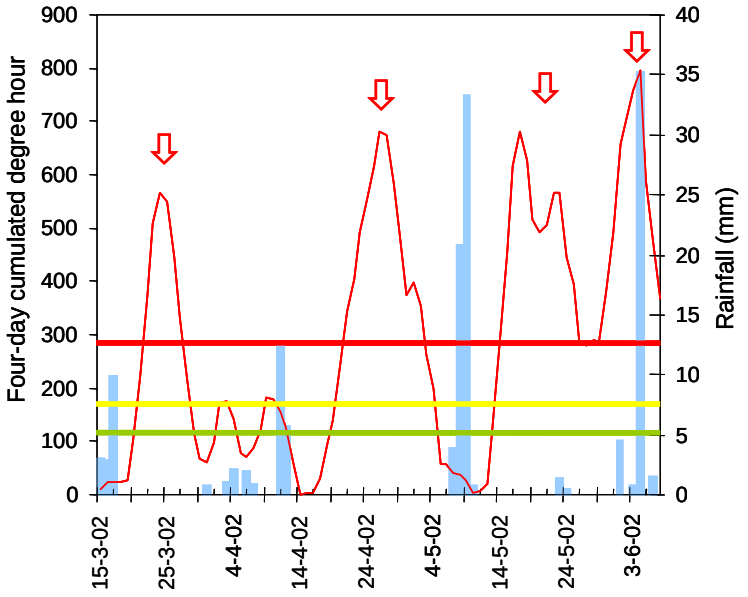


Figure 4.9. Fire blight infection risk predicted by Cougarblight model in Las Llanas, in 2002. Solid line represents the four-day cumulated degree hours, blue bars plot the rainfall. Horizontal lines represent infection risk; under green line: low risk; under yellow line: caution; under red line: high risk, and above red line: extreme risk. Red arrows: extreme infection risk predicted periods.

4. DISCUSSION

In Spain, fire blight only occurs sporadically, mainly in the North of the country, and major economic losses are rare. With the purpose of establishing the probability of fire blight risk to guide disease prospections, we have elaborate fire blight risk maps of Northeast of Spain (Aragón and Catalunya). Two models were used; combined BRS-Powell model, previously developed and used by Montesinos and Llorente (1999) and Llorente *et al.* (2002), and modified BIS95 (Billing, 1996). Results showed two clearly differentiated zones according to fire blight risk, one with high risk around the Ebro river valley (between Huesca and Zaragoza) and Lleida, and the other with low risk including Girona and Barcelona. These results are in agreement with the real distribution of outbreaks appeared in Spain since 1995, which were located in Northeastern Spain, mainly in Euskadi and Navarra. Fire blight outbreaks detected in commercial orchards in Northeast of Spain belong to the high risk area, where weather conditions were favorable to the disease development. Although it should be considered that these results correspond to weather conditions favorable to disease for a given year.

Over the years of study (2000, 2001 and 2002) a trend to diminish the fire blight risk was observed mainly in the area with low risk. These results are also in agreement with observations in orchard, since in the last years no disease outbreaks were detected in Lleida, where the last outbreak had taken place in 1999. Probably as a result of the measures of control carried out along with not favorable weather conditions.

Some differences were found between the two model predictions. The frequency of high risk is higher than could be expected in modified BIS95. Combined BRS-Powell model integrated more information than the modified BIS95, consequently the information displayed by the combined BRS-Powell model is more accurate and realistic. Based on the idea of combined valuable facets of past and current approaches, BRS-Powell model uses concepts from Powell and BRS, with emphasis on increase the strength of the predictions and simplicity and clarity. Combined BRS-Powell model uses mathematical functions to calculate the risk of infection on the basis of weather conditions, and it forecasts periods of possible symptom appearance based on the length, number of disease development periods and data above 18.3 DD. The IRS score alone is not a reliable guide to infection risk because it does not in itself take into account inoculum level or host susceptibility. A low score may still be associated with high risk, or high score associated with low risk, depending on the level of inoculum present, as estimated by the D-period length. Modified BIS95 probably overestimates the fire blight infection risk. Results obtained in the present work seem to indicate that some adjustments must be done in modified BIS95 model, probably in reference to the threshold levels. As our purpose was to simplify the use of the model, we have omitted important information. It is also remarkable that models tested were not adjusted to specific cultivar or species blossom period; consequently some accuracy in disease risk prediction could be lost. The most important feature of Feuerbra model is that it incorporates a factor which changes according to the host susceptibility, and increased length of infection period was obtained when host susceptibility was low, this allowed us to obtain more accurate predictions. Our results indicated that the length of infection period according to Feuerbra was higher than that from BRS. In conclusion, as our objective was to elaborate fire blight risk maps with an overall risk, more than to obtain high accuracy in prediction, Feuerbra was not a valuable model, although it could be useful for the disease prediction in real time in the orchard.

Epidemics of *E. amylovora* occur sporadically between seasons, locations, host species and cultivars and the organs of each host (flower, shoot, stems), as was previously commented, so the evaluation of any fire blight risk assessment system is difficult. As Shtienberg *et al*. (2003) suggested all this difficult the experimentation in fire blight, since the likelihood of occurrence of natural infections with uniform distribution of the disease at an experimental site is low. In our work we evaluated the Maryblyt and Cougarblight models in an orchard in 2002, where the previous season an important fire blight outbreak took place. Results showed that two infection risk periods were predicted by Maryblyt and four by Cougarblight. Both risk periods predicted by Maryblyt model corresponded to the end of the bloom period. The period of favorable conditions for fire

blight development according to the models agreed with the end of blossom period, and with secondary bloom, when the amount of flowers is low. Probably, in years when weather conditions are favorable to host, advanced secondary blossoms could be important in disease development. However, no outbreak had occurred in the orchard, although it is not uncommon that the disease does not develop even in orchards that were severely infected in the preceding season. If we take into account that the previous year (2001) there was an important outbreak in the area of study and eradication measures were accomplished for fire blight control (infected trees were burned), the amount of available inoculum was probably reduced to levels that did not allow the disease development in spite of the favorable weather conditions, as predicted by Maryblyt and Cougarblight models. Moreover, Maryblyt model assumes an abundance of inoculum; consequently it overestimates fire blight risk, predicting infection risk events when in it can not take place because of the absence of the pathogen. In spite of considerable improvements in equipment and methods, assessments of initial inoculum of rapidity of infection cycles are seldom accurate. According to Smith (1999a) most commonly, high infection risk predictions are not followed by actual outbreaks in orchards. Less commonly, fire blight outbreaks occur when no infection was predicted.

Concluding it was not possible to import and successfully implement fire blight warning systems in Spain, which had been developed in regions with dissimilar environmental conditions as also occurs in Israel (Shtienberg *et al*., 2003). Climatic differences between regions presumably influence the capacity of *E. amylovora* to colonize buds, and the disease appearance. Other problem that we found is that low foci of disease took place in the area of study which difficulted the evaluation of the models, and on the other hand records of first signs of disease were not always available.

In order to have a better guidance for inspection or chemical sprays for fire blight control in Spain some adjustments might be needed in the models used (BRS-Powell combined and BIS95 modified) and further research should be done to evaluate the available models (Maryblyt and Cougarblight) in orchards with high incidence of disease. As indicated Billing (1996) ultimately, the value of any system depends on the quality and the quantity of the data input (field and weather) and the knowledge, experience and common sense of the user and all approaches need continual evaluation.

CONCLUSIONS

1) This study has demonstrated that thermotherapy is a viable method for eradicating *Erwinia amylovora* from rosaceous propagation material. However, heat conditions (type of heat, temperature and exposure time) must be established for each host species or cultivar and bacterial strain.

2) Heat sensitivity of propagating plant material depends on the type of cultivar or species, and thermotherapy conditions. Rosaceous species tested have shown low susceptibility to heat. Propagating material has shown good tolerance of exposures of up to 6 hours or 4 hours at 45 ºC under moist or dry heat, respectively; and 3 hours at 50 ºC under moist heat. In general, moist heat was less harmful to plant material than dry heat.

3) *In vitro E. amylovora* thermosensitivity is directly correlated to temperature and exposure time. The time required to reduce bacterial population to a non-detectable level (50 cfu mL^{-1}) was up to 80 min at 45 ºC and up to 60 min at 50 ºC.

4) Epiphytic *E. amylovora* population on pear budwoods are reduced to non-detectable level (5 x 10^2 cfu g^{-1} f.w.) after 60 min at 45 ºC or 30 min at 50 ºC, while endophytic populations of *E. amylovora* in pear plants can be eliminated after 8 hours at 45 ºC or more than 3 hours at 50 ºC.

5) Copper formulations and copper-mancozeb mixtures control similarly the disease in pear plants, and for all products the maximal reduction of disease was obtained at the highest copper-dose (3 g Cu L^{-1}). So, no improvement in their efficacy in fire blight control was achieved. In contrast, the protective application of copper derivatives is more effective than the preventive and curative strategies in control of disease.

6) Phosphonate derivatives and benzothiadiazole are effective in fire blight control in pear and apple, under laboratory, greenhouse and field conditions. Their efficacy depends on the dose and the time interval between product application and bacteria inoculation, since several days are required to completely induce plant defenses. Plant defense inducers reduce disease levels to 40-60%, so they are less effective than, or sometimes similarly effective to antibiotics. The minimal time intervals to achieve the best control of disease are 5 days for fosetyl-Al, and 7 days for ethephon and benzothiadiazole, and the optimal doses of fosetyl-Al and benzothiadiazole are 3.72 g $HPO_3^{2-}L^{-1}$ and 150 mg a.i. L^{-1}, respectively. No phytotoxic effects of fosetyl-Al or benzothiadiazole were observed, at the highest dose tested.

7) The efficacy of fosetyl-Al and benzothiadiazole in fire blight control is improved when combined with a half-reduced dose of antibiotics, achieving the same control level as antibiotics alone. The reduction of antibiotic dose by means of combination with plant defense inducers implies a strategy more environmentally friendly. Simultaneous sprays of chemicals (mixed strategy) are more practical and easier to apply in orchard than the consecutive sprays (combined strategy). However, the best level of fire blight control is achieved with the combined strategy. In this strategy, products are applied at the best time interval, thus optimizing the effect of both compounds.

8) Phosphonates and benzothiadiazole have shown very low *in vitro* antibacterial activity against 14 strains of plant pathogenic and non-plant-pathogenic bacteria tested. This low *in vitro* antibacterial activity together with the moderate efficacy in disease control in pear could make possible their future use in fire blight control.

9) Neither benzothiadiazole, nor fosetyl-Al, nor ethephon induced structural changes in pear leaf tissues 7 days after their application. However, after *E. amylovora* inoculation structural cell disorganization was observed in fosetyl-Al and ethephon-sprayed plants, while in benzothiadiazole-sprayed plants these tissue alterations were delayed.

10) Among some apple molecular markers of SAR only chitinase and β-1,3-glucanase were induced in pear by benzothiadiazole and ethephon, respectively. The study of other PR-proteins and the use of techniques to quantify their expression after the induction could give us further insight into the mechanism of action of benzothiadiazole in pear.

11) Combined BRS-Powell and modified BIS95 models show two clearly differentiated zones according to fire blight risk, one area with high risk around the Ebro river valley (between Huesca and Zaragoza) and Lleida, and an other with low risk including Girona and Barcelona. Over the years of study (2000, 2001 and 2002) the observed fire blight risk tended to diminish. Of the two models, BRS-Powell combined model is the most robust.

12) Maryblyt and Cougarblight are easy models to use, but their implementation in disease management programs must be evaluated and validated over more seasons and in areas where the disease is present.

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