ELSEVIER

Physiological and Molecular Plant Pathology 65 (2004) 305–315

PMPP

www.elsevier.com/locate/pmpp

Extract of *Hedera helix* induces resistance on apple rootstock M26 similar to Acibenzolar-S-methyl against Fire Blight (*Erwinia amylovora*)

Ö. Baysal[a,*], W. Zeller[b]

[a]*West Mediterranean Agricultural Research Institute, P.O. Box 35 07100, Antalya, Turkey*
[b]*Federal Biological Research Centre for Agricultural and Forestry, Institute for Biological Control (BBA), Heinrichstr 243, D-64287 Darmstadt, Germany*

Accepted 4 March 2005

Abstract

The potential of acibenzolar-S-methyl (Benzo [1,2,3]thiadiazole-7-carbothioic acid-S-methyl ester, ASM; Bion 50 WG) and of an extract of *Hedera helix*, to protect M26 apple rootstocks against fire blight was determined under controlled conditions. Marked differences were observed in the rate and extent of multiplication as well as in pathogen cell viability between control and ASM and *H. helix*-treated rootstocks. Although the pathogen multiplied abundantly in the plant tissue of water-treated rootstocks and showed severe damage, ASM and the plant extract of *H. helix* applied prior to inoculation with the causal agent of fire blight, *E. amylovora* (strain 7/74), suppressed disease development and bacterial multiplication. Physiological observations of ASM and plant extract-treated rootstocks indicated that restriction of pathogen colonization in plant tissue was correlated with a pronounced increase of peroxidase (POX) and chitinase activity. Furthermore, physiological changes caused by these treatments in host cells were characterized by POX labeling methods with SDS-Page electrophoresis. Differences in expression of the POX and protein bands were observed in tissues of plants treated with different inducers. POX activity was determined by the presence of three strong bands in plant extract-treated leaves, two strong bands and one very weak band of about 20.1 and 43 kDa were visible in ASM-treated leaves. Evidence is provided that ASM, as well as extract of *H. helix* are equally capable of inducing of resistance responses in M26 apple rootstock, which result in an increased resistance to *E. amylovora*—the fire blight pathogen. These findings demonstrate that both treatments have the ability to induce the activation of defense genes leading to the accumulation of structural and biochemical activities at strategic sites, and these can be associated with induction of resistance against fire-blight.

Keywords: Biological control; ASM and plant extract-mediated induced resistance; *Erwinia amylovora*

1. Introduction

The gram-negative bacterium *Erwinia amylovora* ((Burril) Winslow et al.) is the causal agent of fire blight, an important disease of pome fruits and several *Rosaceae* ornamentals. Fire blight control measures are mostly restricted to the early eradication of infected plants, as in most countries the use of antibiotics is prohibited. In countries where the antibiotic streptomycin has been allowed, strains of the pathogen resistant to streptomycin have been isolated from infected orchards [13,34]. Several chemical copper based compounds, Flumequine, Aliette and Oxolinic acid, have also been tested as alternatives to antibiotics. They either revealed insufficient efficacy under field conditions, or showed negative side effects. Several biological control agents have been developed and are available in some countries. However, their use is limited to preventing blossom blight, and growers would be happy to have in addition to biological control agents a chemical compound which could be used later in the season [33].

Several plant extracts have shown antibacterial activity against *E. amylovora* in vitro and in vivo. In in vitro studies *E. amylovora* was inhibited by 24 of the 139 plant extracts tested by an agar diffusion test [18]. *E. amylovora* was also inhibited with leaf extracts from *Rhus typhina*, *Berberis vulgaris*, and *Mahonia aquifolium* in field experiments [18]. A high activity against the disease was also reported for plant extracts from *Reynoutria sachalinensis*, *Hedera helix*, *Viscum album* and *Alchemilla vulgaris*. These extracts induced resistance in the highly susceptible host plant

* Corresponding author. Tel.: +90 242 3452884; fax: +90 242 3211512.
E-mail address: obaysal@ttnet.net.tr (O Baysal).

0885-5765/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.pmpp.2005.03.003

Cotoneaster waterei, causing a slower multiplication of the bacterium and a reduction in disease severity. In field experiments on the apple variety 'James Grieve', an extract from *H. helix* was as effective as streptomycin in reducing fire blight incidence [24]. Although in vitro growth of the bacteria was not affected by plant extract treatment, foliage sprays of plant extract of *H. helix* significantly suppressed disease development after inoculation at different doses tested, and the most effective concentration was 3% (3 ml plant extract: 100 ml water) against fire blight in field experiments [21,22].

Recently, the benzothiadiazole derivative benzo (1,2,3) thiadiazole-7-carbothioic acid-S-methyl ester (acibenzolar-S methyl, ASM or BTH) has been developed as a systemic acquired resistance (SAR) activator. Such compounds do not have antimicrobial properties, but instead increase crop resistance to diseases by activating the SAR signal transduction pathway [8]. It has been commercially released in some countries as a plant health promoter of annual crops under the name of Bion® or Actigard™. In recent studies, the effect of ASM on the control of fire blight disease and induction of the defense-related enzymes, such as POX and β-1,3-glucanases, in apple were studied [6]. In another study, the induction of two subclasses of PR-10 protein with treatment of ASM, functional analogue of salicylic acid, was determined in apple leaves [40]. The development of SAR is associated with various cellular defense responses, including the synthesis of pathogenesis-related (PR) proteins, phytoalexins, and accumulation of reactive oxygen species (ROS), rapid alterations in cell wall and enhanced activity of various defense-related enzymes [8]. ROS, produced via an oxidative burst, are under control of enzymes such as NADPH oxidase and POXs. POXs have been implicated in the hypersensitive response and the formation of papilla and polymerization of lignin from monomeric lignols. Furthermore, POXs have been implicated in the cross-linking reactions of cell wall associated proteins such as hydroxyproline-rich or glycine-rich glycoproteins [36]. As a result of oxidative cross-linking reactions, cell walls may be strengthened and function as physical barriers against invading pathogens. PR-proteins are arbitrarily divided into eleven groups. The properties of hydrolytic proteins of group 2 (β-1,3-glucanases) and group 3 (chitinases) have been described comprehensively [32]. This is evidence that these enzymes fulfill at least two functions in plant disease control. Thus, they are capable of catalyzing degradation of cell walls of plant pathogenic agents, because β-1,3-glucan and chitin are essential components of the pathogen cell walls and these enzymes catalyze hydrolysis of the corresponding substrates, thereby releasing biologically active oligosaccharides (elicitors and suppressors) capable of regulating the immune status of plant tissues [10].

Little information is available about the physiological changes in apple rootstocks treated with plant extract (PE) from *H. helix* compared to ASM. The aim of this study was to determine the ability of the plant extract to induce resistance in apple plants against *E. amylovora*, and to characterize the changes in activities of peroxidase (POX) and chitinase in rootstocks exhibiting induced resistance following two different treatments.

2. Materials and methods

2.1. Plant material and bacterial strain

The highly susceptible M26 apple rootstock obtained from Rheinau (Deutsche Marken–Baumschule) was used as host plant. Three-month old rootstocks were grown in the greenhouse in pots of 20 cm × 15 cm × 15 cm, filled with 8 kg soil. The temperature was kept at of 25 ± 5 °C, the relative humidity at 68–80%, and the light intensity at 5000–14,000 lux. The plants were used 4 weeks after planting (young shoots were 10–12 cm long with 6–8 leaves per shoot). This environment was maintained during the entire period of the experiment.

The bacterial strain of *E. amylovora* (Ea7/74) was obtained from the Federal Biological Research Centre (BBA), Darmstadt. On M26 rootstocks, Ea7/74 is highly virulent and was used in all the experiments. Stock cultures were preserved on the modified Miller–Schroth medium [19] in glass flasks at 4 °C. The bacteria were transferred every 3 months to new flasks.

2.2. Inoculation and assessment of disease

Inoculum suspension was prepared from early log-phase cells which were obtained by growing the bacterial strain in nutrient yeast extract broth in 25 ml sterile tubes and incubated at 27 °C on an orbital shaker at 200 rpm for 24 h. Bacteria were subsequently pelleted by centrifugation (twice, each at 3500*g* for 5 min) and washed in sterile distilled water (SDW). Concentration was adjusted to 10^8 cfu ml^{-1} by dilution to give OD_{660} of 0.2. For the determination of bacterial multiplication, the two reciprocal youngest leaves of the seedlings were inoculated by dipping into this bacterial solution.

Virulence was assessed using the 0–10 arbitrary scale of [23]. 0—no obvious symptom and minimum necrosis at the cutting point was assessed as no symptom, as it sometimes also occurred in control plants. 1—main leaf vein turned to brown from cutting point, few mm (3–5). 2—main leaf vein turned to brown from cutting point, several mm (>5 mm, not total leaf length) 3—main leaf vein turned to brown from cutting point, total leaf length 5—main and side leaf veins turned to brown and/or necrosis from cutting point, half leaf length 7—total leaf turned to brown and/or necrosis up to leaf stem 10—infection of shoot, often together turning black, and shoot curved. A mean disease severity index (DSI) was calculated from each treatment by summing the score of the plants (two replicates of plants

for each treatment), and expressing the value as a percentage by using the formula described by [9].

2.3. Application of ASM

ASM (Bion®, Syngenta, Frankfurt, as 50% active ingredients in WP formulation) was dissolved in distilled water to obtain concentration of 0.2 mg ml^{-1} and then sprayed on whole seedlings (ca. 200 μl per seedling).

2.4. Preparation and application of the plant extract from H. helix

Dried and ground leaf material (powder and ground leaves of about 3 mm size) supplied by Galke Company (Gittelde/Harz) was used for the preparation of plant extract. The plant material was extracted in 80% methanol with a soxhlet-apparatus. The extract was boiled in 80% methanol for 30 min after cooling with a reflux condenser for 4 h. After 15 min the extract was filtered and dissolved in methanol (1:1 v/w) then again boiled for another 15 min. This treatment was repeated three times. Then, the methanol was evaporated using a rotary evaporator. The plant extract was stored in a refrigerator in 30% ethanol solution until use. Before application, the extracts were diluted with water to 3% extract concentration that for 100 ml of final solution (97 ml of water and 3 ml extract) [21]. The extracts were sprayed on foliage of plants and leaves until run off.

2.5. Effect of delayed inoculation

To determine the most efficient interval between treatment with ASM and fire blight inoculation, apple rootstocks were assigned in equal numbers to treatment groups (10 plants per group). Plants in the first groups were treated with ASM and inoculated with a bacterial suspension 1–4 days after treatment. Plants in the second group were treated with water and inoculated as described (control group). The level of the resistance induced in seedlings against *E. amylovora* was evaluated at 4, 6, 7, 11, 12 and 14 days after inoculation (dai) as described above.

2.6. Effect of ASM and plant extract on growth of E. amylovora in planta

Bacterial colony forming units (cfu) were recovered from inoculated tissues, treated with ASM or plant extract or water 2 days before inoculation, by removing aseptically 5 mm-diameter leaf discs from the region of inoculation. Excised discs were washed in 1 ml of sterile 0.06% NaCl solution (1:1) as described by [38]. From each homogenate, dilution plating (from 10^{-1} to 10^{-6}) was performed on the modified Miller-Schroth (MS) medium and incubated at 27 °C. Aliquots of every other dilution were plated on to MS agar plates. Bacterial colonies were counted after incubation for 48 h. Bacterial numbers in planta were calculated, and a mean value obtained from replicates. Each dilution from each plant shoot was duplicated. Results presented are means for two separate experiments in which three plant shoot were homogenized from each treatment.

2.7. Preparation of samples for determining enzyme activities

Apple rootstocks were assigned in equal numbers to six groups (10 plants per group). Plants in group 1 and 4 were treated with ASM, those in the second and the fifth group was treated with the plant extract and those in the group third and sixth were treated with water. Plants of the first three groups were inoculated with a bacterial suspension 2 days after treatment. From inoculated shoots, tissues were taken at the actual site of inoculation with Ea 7/74. From uninoculated control plants, tissues were taken from sites as similar to those of inoculated shoots. Samples for enzyme extractions were separately taken 1, 2, 4, and 7 days after treatment. To avoid possible effects of leaf cutting, both cut edges (about 2 mm) of the leaf segments were removed and the adjacent tissue was immersed in liquid N_2. The frozen leaf segments were homogenized (1: 5 w/v) in an ice-cold mortar using 50 mM potassium phosphate buffer (pH 7.0) containing 1 M NaCl, 1% polyvinylpyrrolidone, 1 mM EDTA and 10 mM β-mercaptoethanol. Thereafter, the homogenates were centrifuged at 17,000*g* for 20 min at 4 °C and finally, the supernatant (crude enzyme extract) was collected and divided into 1.5 ml portions. When not immediately used for enzyme assays, enzyme extracts were stored at −20 °C. Protein concentrations were determined by the method of [5] using BSA as a standard. The extracts, obtained from two different lots of leaf samples (1 g fresh weight each) for each treatment, were used to determine POX and chitinase activity.

2.8. Enzyme assays

All assays were performed at 25 °C using a UV/visible light spectrophotometer (Uvikon UV1601 PC).

2.8.1. Peroxidase activity

POX activity was determined from the crude extract according to the procedure described by [16]. Guaiacol was used as a common substrate for POXs. The reaction mixture consisted of 0.5 ml of enzyme extract, 0.5 ml of 50 mM sodium acetate buffer (pH 5.6), 0.5 ml of 20 mM guaiacol and 0.5 ml of 60 mM H_2O_2. The linear increase in absorbance at 480 nm resulting from the formation of tetraguaiacol was monitored for 4 min at 30 °C. The enzyme activity was calculated from the change in absorbance and was expressed as mmol of tetraguaiacol produced min^{-1} mg^{-1} protein, using a molar extinction coefficient of 26.6 mM^{-1} cm^{-1}. Sodium acetate buffer was used as a blank. Each sample of extract was measured

twice in each replicate, and at least two replications were performed per analysis.

2.8.2. Chitinase activity

Chitinase activity was determined by the method of [35]. High polymeric carboxymethyl-substituted chitin, labeled covalently with Remazol Brilliant Violet 5R (CM-Chitin-RBV, Loewe, Biochemica, Germany) was used as substrate. Potassium acetate buffer (0.2 ml 0.1 M, pH 5.0) and 0.1 ml of suitably diluted crude extract were added to a micro-centrifuge tube and allowed to equilibrate to 37 °C. The reaction was initiated by adding 0.1 ml aqueous CM-Chitin-RBV (2 mg ml^{-1}), and was terminated by adding 0.1 ml of 2 N HCl, which precipitated the un-degraded substrate. Tubes were cooled on ice for 10 min then centrifuged for 5 min at 9000*g*. Absorbance of the supernatant at 550 nm was recorded and the results were calculated as a change in optical density at 550 nm mg $protein^{-1}$ min^{-1}. Blanks were prepared similarly with Na-acetate buffer instead of the homogenate. Enzyme activity was expressed in micro-molar of the reaction product per minute per milligram protein. Each sample of extract was measured twice in each replicate, and at least two replications were performed per analysis.

2.8.3. Determination of peroxidase activity with staining solution

Two discontinuous SDS slab gel electrophoresis of 15% acrylamide were performed using a vertical mini-gel system (Bio-Rad Inc., USA) with a 0.75 mm thickness for determination of specific POX activity and protein patterns. The electrophoresis tank was filled with 1% SDS puffer (Co. Roth). The samples (5 µl) and standard protein as marker (low molecular weight-marker, Pharmacia calibration Kit; 14.4, 20.1, 30, 43, 67, and 94 kDa) were applied in 1:1 (v/v) sample buffer in well dividers. Electrophoresis was performed at 60 V in the first 2 h, then at 110 V for 1 h more, after which the gels were removed from the electrophoresis tank. One was fixed with 12.5% TCA and stained with coomasie brilliant blue (CBB R-250) dye. The other gel was incubated in staining solution (50 mM sodium acetate buffer, pH 5.0, 100 ml, 3-amino-9-ethyl-carbazole (dissolved in a few drops of acetone), 50 mg 3% H_2O_2 (freshly prepared) 0.75 ml, in the dark at room temperature until red–brown bands appeared [31]. The gel was fixed in 50% glycerol and photographed.

2.9. Experimental design and statistical analyses

All greenhouse experiments were arranged in a completely randomized split-plot design with two replicates of 10 plants for each treatment and repeated at least twice. The Pearson's correlation coefficient was used to account for the direction of the relationship between disease index and days after treatments. A simple multiple linear regression (MLR) was performed to explain variations in the response variable of bacterial growth as a function of the explanatory variables of the treatments and the days after inoculation. Each sample of extract was measured twice in each replicate, and at least four replications were performed per analysis. The significance of differences between mean values was determined. Analysis of variance (ANOVA) was carried out, and the significance of differences among the treatments was determined according to Duncan Analysis ($P < 0.05$).

3. Results

3.1. Reduction of disease incidence in plants treated with ASM

Plants treated with ASM had a disease index of fire blight significantly lower than that of control plants (Fig. 1). The greatest suppression of disease was obtained when plants were treated 48 h before inoculation with *E. amylovora*, although the disease index was also significantly lower for plants treated 72, 96 and 120 h before inoculation. The resistance to *E. amylovora* of ASM-treated plants was first detected 6 days after inoculation and lasted for the entire experiment two weeks after inoculation. Untreated plants showed a significantly faster symptom development during this period. Thus, at 7 days p.i. disease index was reduced by 83–55% in ASM-treated seedlings (ASM was treated 48 and 120 h before inoculation) and at 11 days p.i. 80–62% and 12 days p.i. up to 80 and 57%, respectively. At 14 days p.i., the disease indices of control seedlings were 82% whereas those of ASM-treated seedlings were only 21–35%. Since the highest effect was observed at a time interval of 48 h between treatment and inoculation, this delay was kept for all further experiments.

3.2. Reduction of disease incidence in plants treated with H. helix plant extract

Application of the *H. helix* plant extract (PE) significantly reduced symptom development on apple seedlings. However, stable differences between different time intervals were not observed. Therefore, the same induction time (48 h before inoculation) as for ASM-treatment was also chosen for application of PE At 4 days p.i., treatment with ASM and PE reduced the disease index by 40%, although not statistically significant (Fig. 2). Six days after inoculation, the PE and ASM-treated seedlings showed 50 and 58% reduction in symptom development, respectively. At all the following dates, from 7 to 14 days post-inoculation, ASM-treatment always reduced disease indices more than treatment with plant extracts (Fig. 2).

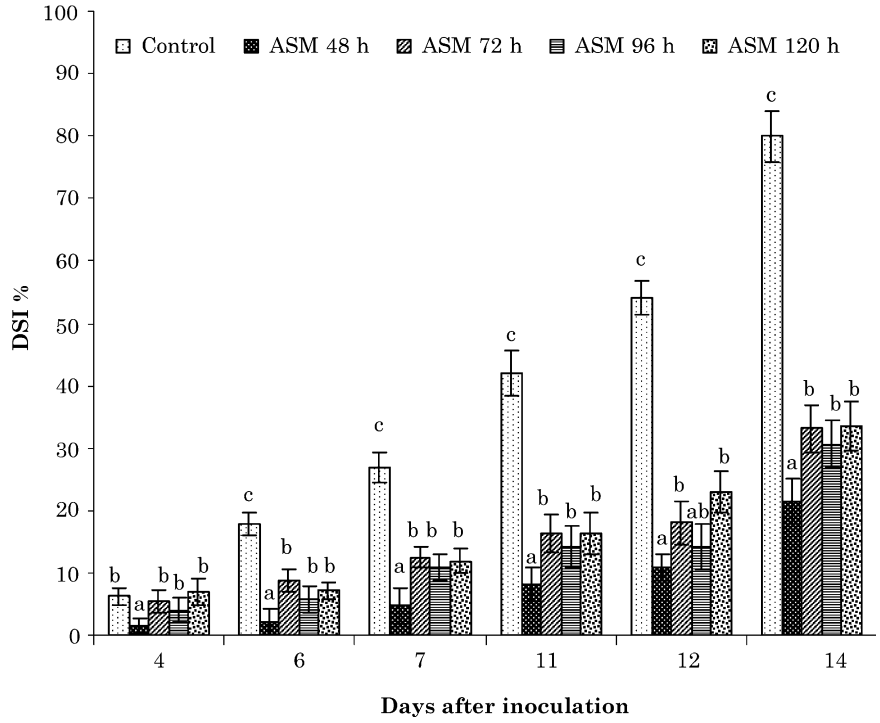

Fig. 1. The M26 rootstocks were inoculated with the Ea 7/74 and the plants were treated with ASM, or water (control) 48 h before inoculation. Disease index of fire blight on M26 rootstocks after different time intervals between ASM-treatment and inoculation (48–120 h) were investigated. Disease symptom was evaluated at 4 and 14 days after inoculation (dai). Disease index (DI) was calculated from each treatment by summing the score of the 10 plants (two replicates of 10 plants per treatment) by using the 0–10 scale as described in Section 2. The values followed by different letter are significantly different according to Duncan test ($P<0.05$). Standard deviations of disease index for two replicates (10 plants per variant).

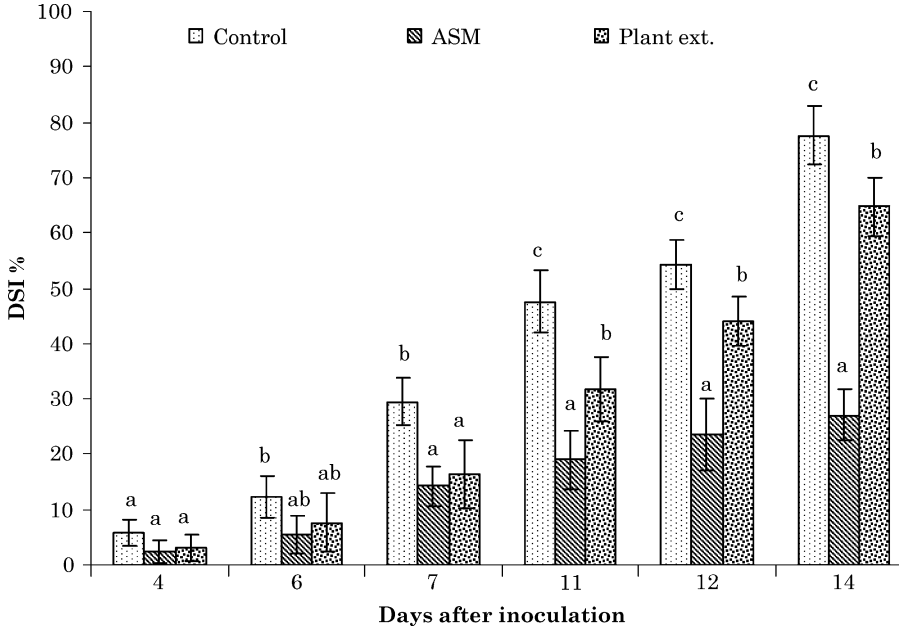

Fig. 2. The M26 rootstocks were inoculated with Ea 7/74 and the plants were treated with ASM, PE or water (control) 48 h before inoculation. Disease symptom was evaluated at 4 and 14 days after inoculation (dai). Disease severity index (DSI) was calculated from each treatment by summing the score of the plants (two replicates of 10 plants per treatment) by using the 0–10 scale as described in Section 2. The values followed by different letter are significantly different according to Duncan's Multiple Range Test ($P<0.05$). Standard deviations of disease index for two replicates (10 plants per variant).

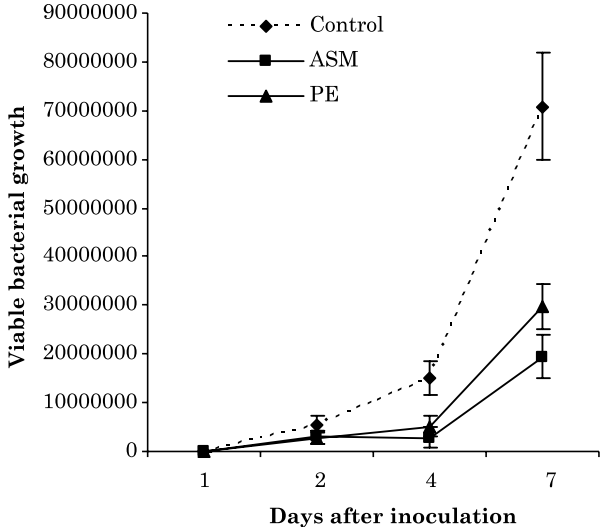

Fig. 3. The effect of ASM and PE treatment on the growth of *Ea 7/74* in M26 rootstocks. Two days before inoculation the rootstocks were treated with ASM, PE or water. Data are the mean of two independent experiments, and the values represent standard deviations. Effect of the treatments on bacterial growth was significant according to student's two-sample *t* test ($P<0.05$).

3.3. Growth of *E. amylovora* in plants treated with ASM or plant extract

The growth of *E. amylovora* was markedly reduced in ASM and PE-treated seedlings compared to the untreated control (Fig. 3). The inhibitory effect was first observed at 2 days after inoculation and was still significant at 7 days after inoculation. ASM-treatment caused a stronger reduction of bacterial growth than treatment with PE. The bacterial population was reduced by 80 and 65% by ASM, compared to only 72 and 58% by plant extracts at 4 and 7 days after inoculation, respectively (Fig. 3).

3.4. Peroxidase (POX) activity

In the uninoculated plants, over the entire experimental period, POX activity was markedly increased in ASM (42%) and PE (54%) treated tissue (Fig. 4), starting 2 days after induction with a significantly higher activity. At 4 days after induction, the plant extract treated shoots showed a significantly higher activity than ASM treated ones (67%). In PE-treated plants, the highest increase was up to 135%. Afterwards, the POX activity of PE-treated plants gradually decreased although; it was also significantly higher than ASM-treated plants at 7 days after treatment though ASM-treated plants showed higher activity than water-treated plants.

The course of POX activity in inoculated plants was different from that in uninoculated plants (Fig. 4). One day after treatment, POX activity was significantly lower in ASM-treated inoculated plants than in PE-treated inoculated and control (water-treated) inoculated plants. The activity increased to 50% greater than water-treated plants (control)

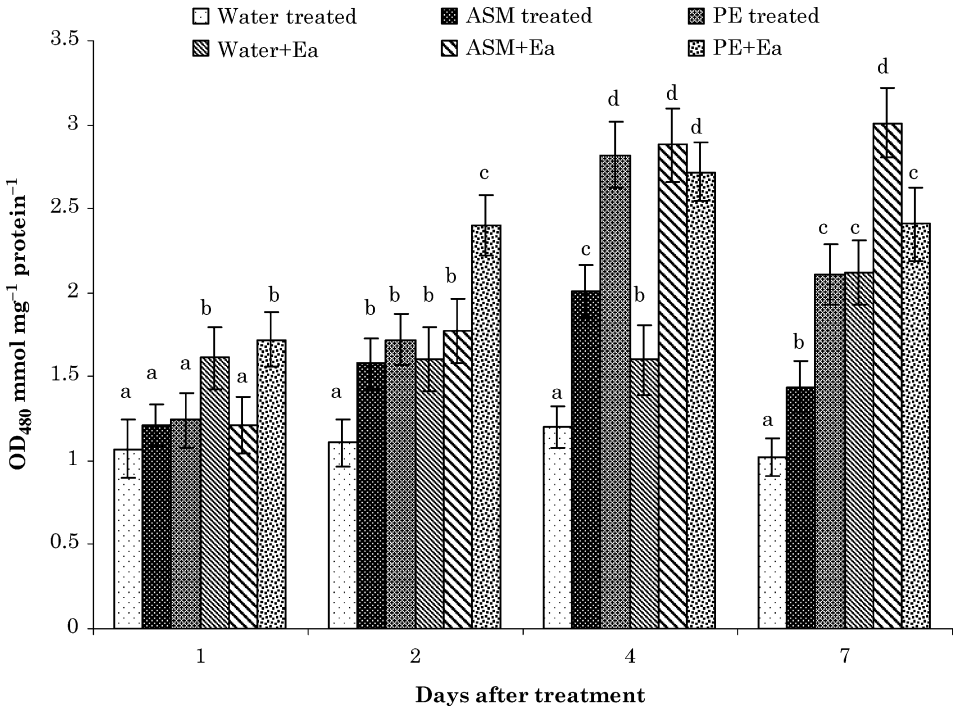

Fig. 4. The effect of the ASM and PE treatment on the induction of POX activity in the shoot leaves of M26 rootstocks. Two days before inoculation, the seedlings were treated with ASM, PE or water. For controls, the rootstocks were not inoculated but sprayed with water or ASM and PE. Both inoculated (Ea: *Erwinia amylovora* infected) and uninoculated shoots were removed at the indicated periods and processed as described in Section 2. The results are expressed as the mean of two separate experiments (in each experiment two different extractions were pooled at every time point). Bars with the same letters represent values that are not significantly different according to Duncan's Multiple Range Test ($P<0.05$).

in PE-treated plants at 2 days after treatment, and was significantly higher than in ASM treated and control plants. The activity in control plants remained on the same level up to 4 days after treatment. In PE treated plants, the highest activity (70% more than the control) was observed at 4 days after treatment, and also ASM-treated plants showed a similar activity (80% of control) at the same time. At the end of the experimental period (7 days) in ASM treated plants the activity was at the highest level. POX activity of PE-treated inoculated plants was to that found in PE-treated, uninoculated plants.

3.5. Specific peroxidases and protein patterns detected by electrophoresis

To characterize specific changes in POX, the period of highest activity (4 days after treatment) was examined by SDS-PAGE. As demonstrated in Fig. 5a, POX activities were found at protein sizes of 20.1 and 43 kDa. POX activity in water-treated plants was very slightly detectable at molecular weight of nearly 43 kDa as one band on SDS-Page, but two bands showed slightly detectable expression at molecular a weight of about 43 and 20.1 kDa in ASM-treated and PE-treated uninoculated plants, respectively. The PE-treated inoculated plants showed high POX activities with three bands (between 20.1 and 43 kDa) and there was strong expression in ASM-treated inoculated plants with two bands, and one band showed low expression at 4 days (Fig. 5a). These results correlated reasonably well with the spectrophotometrical measurements of POX activity in inoculated plants. The period of highest activity (4 days after treatment) was examined by SDS-PAGE and proteins bands were characterized in water-treated, ASM-treated and PE-treated plants (Fig. 5b). Protein bands were recorded 4 days after treatment. In the samples of ASM and plant extract-treated seedlings, a protein band about of 43 kDa appeared to increase in intensity. In the homogenate of ASM-treated plants, additional protein bands at 43–20.1 kDa were observed, which were not present in the water-treated plants. The treatment with plant extract induced high intensity of specific protein bands similar to the pattern of inoculated water-treated plants (Fig. 5b).

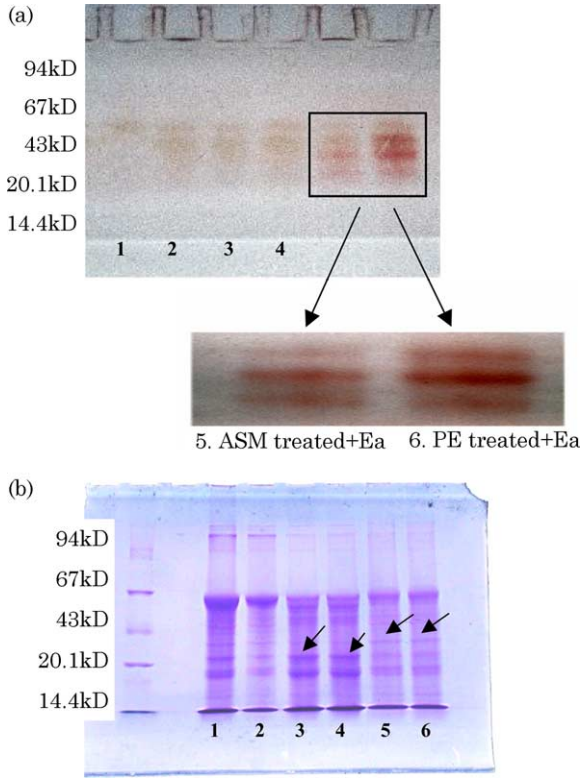

Fig. 5. (a) At 4 days after treatment specific peroxidase activities of ASM and plant extract treated shoots on SDS-PAGE Gel Electrophoresis (1 g plant material per sample). The experiments were independently repeated two times. (1) Water-treated shoots. (2) ASM treated. (3) PE treated. (4) Water + Ea 7/74. (5) ASM + Ea 7/74. (6) PE + Ea 7/74. (b) At 4 days after treatment, protein bands of ASM and plant extract treated shoots on SDS-PAGE Gel Electrophoresis (1 g plant material per sample). The experiments were independently repeated two times. (1) Water-treated shoots. (2) ASM-treated. (3) PE-treated. (4) Water + Ea 7/74. (5) ASM + Ea 7/74. (6) PE + Ea 7/74.

3.6. Chitinase activity

Chitinase activity in ASM-treated plants was higher than in control and PE-treated plants with exception of the first stage after treatment (1 and 2 days) (Fig. 6). The activity increased from 1 to 4 days after treatment in ASM-treated shoots up to 138% and in PE-treated shoots up to 88% compared to the control. While in ASM-treated plants, the activity was considerably higher than in water-treated plants during the whole experimental period, a sharp decrease was recorded in plant extract-treated plants at 4 days after induction in control shoots. At the end of the experiment (7 days), there were differences in the activity of ASM and PE-treated and inoculated variants.

In inoculated shoots, chitinase activity was generally higher than in uninoculated plants (Fig. 6). Enzyme activity increased in ASM and PE-treated shoots from 1 to 4 days post-induction and was higher in ASM treated (214% of the control) than in PE-treated (151% of the control) plants. The highest activity was observed in ASM and PE-treated plants at 4 days after treatment. In all three variants, there was a drop in chitinase activity after 4 days until the end of the experiment. Chitinase activity in water-treated inoculated plants remained at the same level up to 4 days p.i. with a slight increase at end of the experiment.

4. Discussion

The current study assessed the effect of plant extract of *H. helix* and the plant activator ASM on the disease development by *E. amylovora*. Induction of resistance by ASM has already been demonstrated in a number of plant

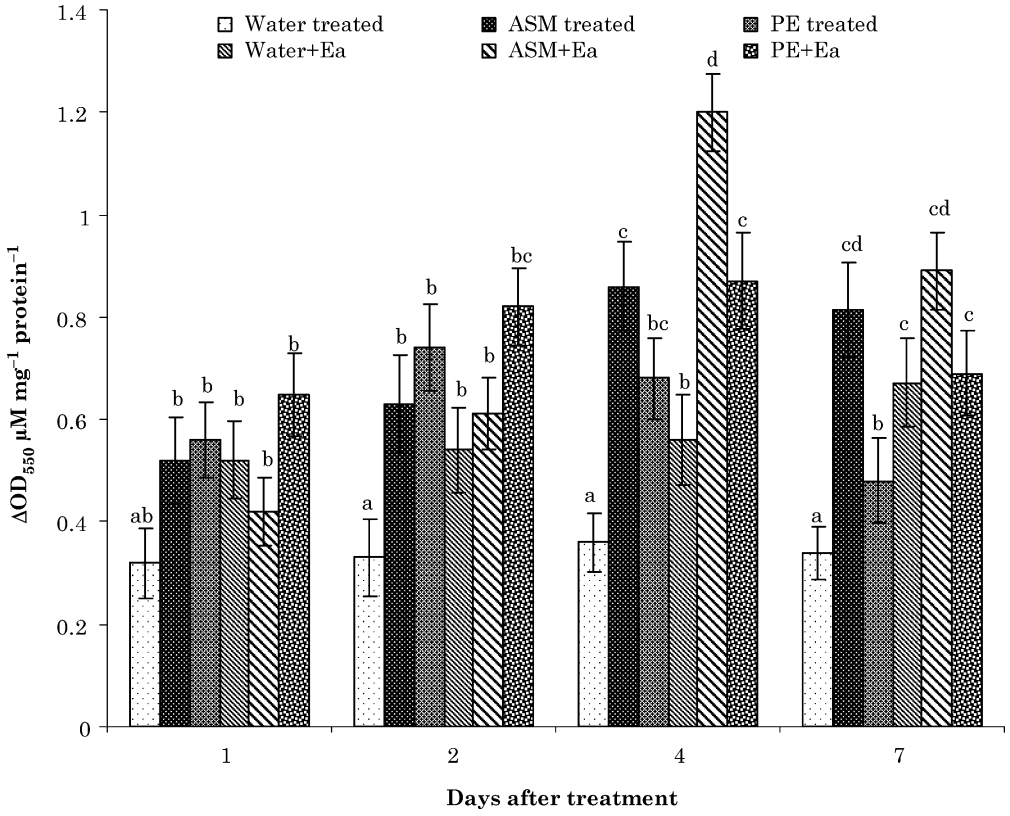

Fig. 6. The effect of the ASM and PE treatment on the induction of chitinase activity in the shoot leaves of M26 rootstocks. Two days before inoculation the seedlings were treated with ASM, PE or water. For controls, the rootstocks were not inoculated but sprayed with water or ASM and PE. Both inoculated (Ea: *E. amylovora* infected) and uninoculated shoots were removed at the indicated periods and processed as described in Section 2. The results are expressed as the mean of two separate experiments (in each experiment two different extractions were pooled at every time point). Bars with the same letters represent values that are not significantly different according to Duncan's Multiple Range Test ($P<0.05$).

species against a wide spectrum of fungal, bacterial and viral pathogens [6,9,28]. The plant extract (PE) of *H. helix*, which has been tested for 10 years showed the highest suppression effect against fire blight in field experiments when compared to other plant extracts [20–23]. In our previous study [2], *H. helix* caused local induced resistance, resulting in significant increase of polyphenol oxidation activity in leaves of M26 rootstocks, and was found at a higher level than in inoculated water-treated plants. Therefore, a different mechanism of action can be assumed for PE.

For the development of resistance, plants need some time before being challenged with a pathogen. In most cases, this interval was reported to lie between 1 and 7 days. In our study, the best protection against *E. amylovora* was obtained when ASM was applied 2 days before inoculation. Although later challenge gave higher disease protection, the best protection was achieved 2 days before inoculation in ASM-treated plants compared to water-treated ones. This result did not compare to findings of [9] and [15] in the cauliflower and cashew pathosystems. According to [28], and our previous studies [3] conducted on tomato a minimum of 96 and 72 h was found to be an efficient interval between application of acibenzolar-S-methyl and inoculation of leaves against fungal and bacterial pathogens. Reduction of fire blight incidence in ASM and PE-treated plants correlated well with a significantly lower bacterial growth in treated plant leaves. In this study PE and ASM significantly reduced bacterial population at 96 h after inoculation compared to untreated plants. Experiments with a diverse set of fungal species showed that neither ASM nor its major metabolites exhibit antimicrobial activity in vitro even at concentrations exceeding the levels shown to be efficacious in plants [9]. In tomato, bacterial growth of *Pseudomonas syringae* pv. *tomato* was slightly inhibited by ASM in vitro as reported by [27] suggesting that the protection of tomato seedlings from pathogens must have been due mostly to the activation of plant defense mechanisms. Reduced bacterial growth also was reported in bean, pepper, tobacco and tomato plants treated with ASM [3,7,28]. Treatments of Arabidopsis and tomato with ASM have also resulted in resistance to Turnip crinkle virus and CMV [1,14]. It was shown that the resistance induced in these plants markedly reduced the replication of viral RNA.

To understand the mechanism of action of ASM and PE, alterations in the levels of two key defense-related enzymes, POX and chitinase, were evaluated. ASM has been found to be an elicitor of the defense pathway in tomato, inducing

remarkable activities of POX and chitinase, enzymes which play a key role in disease resistance against a variety of pathogens [3]. In previous studies, a correlation was found between the activation of defense-related enzymes such as POX, β-1,3-glucanase and reduction in bacterial growth of *Erwinia amylovora*. In our present study, a correlation was found between ASM and PE treatment and increase of POX activity, shown as a key enzyme responsible for generation of reactive oxygen species plants [36]. Induction of POX has been implicated in production of toxic radicals, such as O^{-2}, and H_2O_2, The increased production of both the superoxide radical and H_2O_2 is a common feature of defense responses to plants challenged by avirulent pathogens and elicitors and there is ample evidence indicating that H_2O_2 performs several important functions in disease resistance [17]. The reinforcement of the plant cell wall by phenolics and lignin increases plant resistance to wall degrading enzyme and toxins produced by pathogens, and acts as mechanical barrier to physical penetration toward the protoplast [25]. POX increase in PE-treated and ASM-treated plants may cause oxidative cross-linking of proteins to increase the resistance against bacterial pathogen. Interestingly, it may be hypothesised that a low nutrient concentration or/and accumulation of antimicrobial compounds in the intercellular spaces of treated apple tissues, where bacteria grow, or cell wall alterations such as a physiological barriers in xylem tissues may be a limiting factor for bacterial growth as a consequence of the PE treatment, and this may be responsible for the reduction in disease severity as in the case of other studies. Treatment of apple shoots with inducers caused a general increase of POX activity, especially after PE application. POX activity has been associated with induced resistance after inoculation with several pathogens and especially acidic POX in the cell wall [29]. Increase in POX activity can be involved in the formation of lignin and inhibition of the pathogen's spread in the xylem [37]. In our studies, a detectable increase in three acidic POXs was found after inoculation in ASM and PE-treated shoots. The POX isoenzymes detected in ASM and PE-treated seedlings appear to be associated with induced resistance. However, the number of POX bands after SDS-PAGE differed. Therefore, POX and chitinase activities were increased, it is suggested that activation of isoenzymes and the mechanism of POX activity is different between ASM-treated and PE-treated apple seedlings. In PE-treated shoots, it appeared as if the same proteins were expressed as after artificial inoculation. However, in the ASM-treated shoots different protein bands showed a high expression compared to water-treated and PE-treated shoots. Therefore, during development of induced resistance it is possible that ASM and PE may be recognised by the plant as elicitors. Therefore, ASM and PE may trigger defence mechanisms in the plant and also affect the production of some antibacterial substances by increasing POX activity. However, understanding of the signals and function of POX and the biochemical processes underlying these cytological changes is very poor. It has been demonstrated that in some cases specific POX isoenzymes increase in the host tissues in response to pathogen attack. [11] showed at least three POX isoforms associated with induced resistance. A similar set of acidic POXs was shown in watermelon and muskmelon at molecular weights of 30–33 kDa [29]. Later, [26] reported a 33 kDa apoplastic POX in systemic induced resistance. In SDS-PAGE a few protein bands were detected, which increased after application of the elicitors. This response may include both phytoalexin synthesis and the direct effect of hydrolytic activities. In POX labeled SDS-PAGE, a few protein bands were detected, which increased after application of the elicitors. Concerning POX activity in water-treated plants, one band was just detectable at a molecular weight of nearly 43 kDa on POX labeled SDS-Page, but two bands showed slight expression in ASM-treated and PE-treated plants at a molecular weight of 43 and 20.1 kDa. However, ASM and PE-treated inoculated plants showed stronger POX expression than uninoculated and inoculated water-treated plants. Interestingly, in PE and ASM-treated uninoculated plants although POX activity was found to be significantly higher than water-treated uninoculated plants, it can be assumed that the appearance of differences in bands may be associated with the activation of pathogen specific POX isoenzymes induced in plant after the treatments, which are not present before inoculation on POX labeled SDS-PAGE. These protein bands may be assumed to represent specific PR proteins activated only by the presence of the pathogen.

The enzymatic activities of several PR proteins have been identified and include -1,3-glucanases (PR-2) and chitinases (PR-3), which posses direct antimicrobial activity by degrading microbial cell wall components [30] and chitinases which have lysozyme activity and can therefore hydrolyse bacterial cell walls [4,12]. Therefore, contrary to the findings of [6], in present study the activity of chitinase was evaluated and we found a correlation between resistance and the accumulation of chitinase activity. Induction of PR protein chitinase in ASM-treated and plant extract-treated leaves agrees with the finding of [14] who also found increased accumulation of chitinase and glucanase in Arabidopsis and tobacco. Our results, however, contrast with those obtained by [39], who showed that ASM did not induce accumulation of chitinase in cauliflower inoculated with *P. parasitica*. In this case, an increase in chitinase activity may be accompanied by lysozyme activity against pathogenic bacteria. It is possible therefore that PE and ASM treatments induce a difference in the level of chitinase activity, which differ in response to bacteria and water-treated seedlings.

In conclusion, the plant extract of *H. helix* and ASM are responsible for a stimulation of resistance to fire blight in the host plant and can accelerate the defense response to stop bacterial migration in the plant tissue for up to 7 days. As in other host–parasite interactions, the plant extract of

H. helix caused induced resistance against fire blight on M26 apple rootstocks.

Acknowledgements

The authors thank Joel L. Vanneste for his help with the preparation of the manuscript.

References

[1] Anfoka GH. Benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester induces systemic resistance in tomato (*Lycopersicon esculentum* Mill. cv. volledung) to cucumber mosaic virus. Crop Prot 2000;19: 401–5.

[2] Baysal Ö, Laux P, Zeller W. Studies on induced resistance (IR) effect of the plant extract (*Hedera helix*) against fire blight. In: APS plant pathology meeting, international conference in plant pathology Caribbean division in Cuba p. 82.

[3] Baysal Ö, Soylu ME, Soylu S. Induction of defense related enzymes and resistance by the plant activator acibenzolar-S-methyl in tomato seedlings against bacterial canker caused by *Clavibacter michiganensis* spp. *michiganensis*. Plant Pathol 2003;52:747–53.

[4] Boller T, Gehri A, Mauch F, Vogeli U. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 1983;157:22–31.

[5] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:248–50.

[6] Brisset MN, Cesbron S, Thomson SW, Paulin CP. Acibenzolar-S-methyl induces the accumulation of defence-related enzymes in apple and protects from fire blight. Eur J Plant Pathol 2000;106:529–36.

[7] Buonaurio R, Scarponi L, Ferrara M, Sidoti P, Bertona A. Induction of systemic acquired resistance in pepper plants by acibenzolar-S-methyl against bacterial spot disease. Eur J Plant Pathol 2002;108:41–9.

[8] Conrath U, Thulke O, Katz V, Schwindling S, Kohler A. Priming as a mechanism in induced systemic resistance of plants. Eur J Plant Pathol 2001;107:113–9.

[9] Godard JP, Ziadi S, Monot C, Le Corre D, Silue D. Benzothiadiazole (BTH) induces resistance in cauliflower (*Brassica oleracea* var *botrytis*) to downy mildew of crucifers caused by *Peronospora parasitica*. Crop Prot 1999;18:397–405.

[10] Hahlbrock K, Scheel D, Logemann E, Nürnberger T, Parniske M, Reinold S, et al. Oligopeptide elicitor-mediated defence gene activation in cultured parsley cells. Proc Natl Acad Sci USA 1995; 92:4150–7.

[11] Hammerschmidt R, Nuckles EM, Kuć J. Association of enhanced peroxidase activity with induced resistance systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiol Plant Pathol 1982; 20:73–82.

[12] Heitz T, Segond S, Kauffmann S, Geoffroy P, Prasad V, Brunner F, et al. Molecular characterization of a novel tobacco pathogenesis-related (PR) protein—a new plant chitinase/lysozyme. Mol Gen Genet 1994;245:246–54.

[13] Jones AL, Schnabel EL. The development of streptomycin resistant strains of *Erwinia amylovora*. In: Vanneste JL, editor. In fire blight, the disease and its causative agent *Erwinia amylovora*. Wallingford, UK: CABI Publishing; 2000. p. 235–52.

[14] Lawton KA, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H, et al. Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. Plant J 1996;10:71–82.

[15] Lopez AMQ, Lucas JA. Effects of plant defence activators on anthracnose disease of cashew. Eur J Plant Pathol 2002;108:409–20.

[16] Maehly AC, Chance B. Methods of biochemical analysis. vol. 1. New York: Interscience Publishers, Inc.; 1954.

[17] Mehdy MC, Sharma YK, Sathasivan K, Bays NW. The role of activated oxygen species in plant disease resistance. Physiol Plant 1996;98:365–74.

[18] Mende A, Mosch J, Zeller W, et al. Untersuchungen zur Resistenzinduktion durch ausgewählte Pflanzenextrakte gegen den Feuerbrand (*Erwinia amylovora* (Burril) Winslow et al.). Z Pflanzkrankh Pflschutz 1993;101:141.

[19] Miller TD, Schroth MN. Monitoring the epiphytic populations of *Erwinia amylovora* on pear with selective medium. Phytopathology 1972;62:1175–82.

[20] Mosch J, Zeller W. Control of fire blight with plant extracts based on resistance induction. Acta Horticult 1989;489:577–82.

[21] Mosch J, Zeller W. Praxisorientierte Versuche zum Einsatz von Pflanzenextrakten mit resistenzinduzierenden Eigenschaften gegenüber Bakteriosen. Arbeitsbericht 1997;5:40 ['Biologischer Pflanzenschutz durch Induzierte Resistenz', Biologische Bundesanstalt für Land und Forstwirtschaft].

[22] Mosch J, Klinghauf F, Zeller W. On the effect of plant extracts against fire blight (*Erwinia amylovora*). Acta Horticult 1989;273:355–61.

[23] Mosch J, Mende A, Zeller W, Rieck M, Ulrich W. Plant extracts with a resistance induction effect against fire blight (*Erwinia amylovora*). Acta Horticult 1993;338:389–95.

[24] Mosch J, Zeller W, Rieck M, Ulrich W. Further studies on plant extracts with a resistance induction effect against *Erwinia amylovora*. Acta Horticult 1996;411:361–6.

[25] Nicholson RL, Hammerschmidt R. Phenolic compounds and their role in disease resistance. Annu Rev Phytopathol 1992;30:369–89.

[26] Rasmussen JB, Hammerschmidt R, Zook MN. Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. syringae. Plant Physiol 1991;97:1342–7.

[27] Scarponi L, Buonaurio R, Martinetti L. Persistence and translocation of a benzothiadiazole derivative in tomato plants in relation to systemic acquired resistance against *Pseudomonas syringae* pv. *tomato*. Pest Manag Sci 2001;57:262–8.

[28] Siegrist J, Glenewinkel D, Kolle C, Schmidtke M. Chemical induced resistance in green bean against bacterial and fungal pathogens. J Plant Dis Prot 1997;104:599–610.

[29] Smith JA, Hammerschmidt R. Comparative study of acidic peroxidases associated with induced resistance in cucumber, muskmelon and watermelon. Physiol Mol Plant Pathol 1998;33:255–61.

[30] Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoghi S, Kauffmann S, Geoffroy P, et al. Plant 'pathogenesis-related' proteins and their role in defence against pathogens. Biochemistry 1993;75: 687–706.

[31] Vallejos CE. Enzyme staining. In: Tanskley SD, Orton TJ, editors. Isoenzymes in plant genetics and breeding. Part A. Amsterdam: Elsevier; 1983. p. 469.

[32] Van Loon LC. Induced resistance in plants and the role of pathogenesis-related proteins. Eur J Plant Pathol 1997;103:753–65.

[33] Vanneste JL. Honey bees and epiphytic bacteria to control fire blight, a bacterial disease of apple and pear. Biocontrol News Inf 1996;17: 67–78.

[34] Vanneste JL, Voyle MD. Characterisation of transposon, genes and mutations which confer streptomycin resistance in bacterial strains isolated from New Zealand orchards. Acta Horticult 2001;590:493–5.

[35] Wirth SJ, Wolf GA. Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. J Microbiol Methods 1990;12:197–205.

[36] Wojtaszek P. The oxidative burst: a plant's early response against infection. Biochem J 1997;322:681–92.

[37] Zeller W. Untersuchungen zur Feuerbrandkrankheit in der Bundesrepublik Deutschland. Habilitationsschrift.: Universität Hannover; 1985.

[38] Zeller W, Brulez W. Changes in the phenol metabolism of ornamental shrubs (Cotoneaster species) infected with *Erwinia amylovora*. In: Proceedings of the sixth international conference on plant pathology bacteria Beltsville, Maryland, USA p. 686–94.

[39] Ziadi S, Barbedette S, Godard JF, Monot C, Le Corre D, Silue D. Production of pathogenesis-related proteins in the cauliflower (*Brassica oleraceae* var. *botrytis*)-downy mildew (*Peronospora parasitica*) pathosystem treated with acibenzolar-S-methyl. Plant Pathol 2001;50:579–86.

[40] Ziadi S, Poupard P, Brisset MN, Paulin JP, Simoneau P. Characterization in apple leaves of two subclasses of PR-10 transcripts inducible by acibenzolar-S-methyl, a functional analogue of salicylic acid. Physiol Mol Plant Pathol 2001;59: 33–43.