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Acibenzolar-S-methyl induces the accumulation of defense-related enzymes in apple and protects from fire blight

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Abstract

Acibenzolar-S-methyl (Novartis) is a chemical inducer of systemic acquired resistance in several annual plants. The ability of this novel chemical to induce resistance was studied in a perennial plant (apple) affected by fire blight caused by *Erwinia amylovora*. Acibenzolar-S-methyl (100 and 200 mg/l active ingredient) protected Golden Delicious seedlings, scions and trees from artificial infection when applied before inoculation. The protection of apple seedlings was similar to the protection obtained with the standard for fire blight control, plantomycin (100 mg/l streptomycin sulfate), applied immediately before inoculation. The mean levels of control in scions in the greenhouse and in trees in orchards were approximately 69% and 50%, respectively. The protection of apple seedlings was constantly associated with the activation of two families of defense-related enzymes, peroxidases and β-1,3-glucanases. Accumulation of both enzymes was induced locally in treated leaves and systemically, especially for β-1,3-glucanases, in upper untreated leaves, and was sustained for at least 17 days. These results suggest that acibenzolar-S-methyl promotes induced systemic resistance in apple by increasing defense-related compounds. This chemical could provide a new approach of control of fire blight but its practical use needs further investigation.

Abbreviation: ASM – acibenzolar-S-methyl.

Introduction

Fire blight is a disease of *Maloideae* (apple, pear, ornamentals) caused by the bacterium *Erwinia amylovora*. Pathogen invasion occurs primarily via natural openings in flowers (nectarthodes) or through wounds on young aerial vegetative parts. When established, bacteria multiply and progress in the intercellular spaces between parenchyma cells, leading to the rapid development of necrosis of infected tissues, and to the production of ooze, which are the typical symptoms of the disease (Thomson, 1992). Chemical control relies upon the use of antibiotics (such as streptomycin) and copper compounds which prevent bacterial multiplication and further infection. Unfortunately, the antibiotics have lead to the selection of resistant bacterial populations and therefore their use is strictly limited or even forbidden in a number of countries. The copper compounds usually cause phytotoxicity on fruit finish and therefore cannot be used for high quality fruit (Paulin, 1996). In addition, none of these chemicals is systemic, and to be effective, they have to be applied with thorough coverage before the pathogen enters the plant tissues.

An emerging strategy in plant protection is the chemical induction of systemic acquired resistance (SAR) (Lucas, 1999). 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 systemically 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. Various inorganic and organic compounds have been described as SAR inducers. Among them, acibenzolar-S-methyl (CGA 245704 or benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester), referred to as ASM, is a synthetic molecule from Novartis whose role as a plant defense activator has been demonstrated in a number of annual plants including *Arabidopsis* (Lawton et al., 1996), tobacco (Friedrich et al., 1996), wheat (Görlach et al., 1996), bean (Siegrist et al., 1997), maize (Morris et al., 1998) and cucumber (Narusaka et al., 1999). It is commercially released in some countries as a plant health promoter of annual crops under the name of Bion® or Actigard®.

There have been only few reports on chemical induction of resistance in fruit trees. Another chemical inducer, 2,6-dichloroisonicotinic acid (referred to as INA) provides good levels of protection against pear fire blight (Kessmann et al., 1994) and against apple scab (*Venturia inaequalis*) (Ortega et al., 1998). Concerning ASM, this compound has only been tested on Japanese pear (*Pyrus pyrifoliae*) and was found effective against rust (*Gymnosporangium asiaticum*) and scab (*Venturia nashicola*) (Ishii et al., 1999). These different studies suggest that systemic induced resistance can also be triggered in perennial crops such as fruit trees.

In this paper, the potential of ASM to protect apple seedlings, scions and trees from fire blight infection was investigated. The analysis of two types of defense-related enzymes, peroxidases (EC 1.11.1.7) and β-1,3-glucanases (EC 3.2.1.39), was also undertaken in apple tissue, to assess a possible relationship between the activation of these enzymes and the observed protection. Preliminary results on protection from fire blight of apple induced by ASM and on activation of some defense-related enzymes have been presented elsewhere (Thomson et al., 1998a,b).

Materials and methods

Biological material

Greenhouse experiments were performed on apple seedlings (2–10 leaves) from open-pollinated cv. Golden Delicious, and on 2-year-old scions of Golden Delicious grafted on M9 rootstocks. Plants were grown in individual pots in the greenhouse at temperatures comprised between 17 and 22 °C, under natural photoperiods during the growth season and supplemented with artificial light in fall and winter. The orchard trials were conducted in a block of adult Golden Delicious (4th leaf at the beginning of the experiment) grafted on M9 rootstock. A virulent French standard strain CFBP1430 of *E. amylovora* (Paulin and Samson, 1973) was used for the inoculations. Bacteria were grown for 24 h at 27 °C on King's medium B (King et al., 1954). Bacterial suspensions were prepared in sterile distilled water to yield a concentration of 10^7, 10^8 or 10^9 colony-forming units (CFU) per ml according to the experiment and used within 30 min.

Plant protection experiments

In the greenhouse, apple seedlings were sprayed once with ASM (50% water dispersible granules) at 100 or 200 mg l^{-1} active ingredient 10–2 days prior to inoculation. On the day of inoculation, the youngest nearly expanded leaf of each plant was wounded by making two cuts, 1 cm long across the midrib, and the bacterial suspension (10^8 CFU ml^{-1}) was sprayed on the entire seedling 4 h later. As controls, untreated seedlings were either just wounded and then inoculated 4 h later, or sprayed with the standard streptomycin sulfate 100 mg l^{-1} (plantomycin 17.76% active ingredient) immediately after wounding and allowed to dry for 4 h before inoculating. Development of fire blight symptoms (petiole and shoot necrosis) was assessed within 3 weeks after inoculation. In each experiment, 3 repetitions of 10 plants were used per treatment, and the experiments was repeated 2 times.

Potted Golden Delicious scions were sprayed twice with ASM at 100 mg/l active ingredient (or water for the control) 7 and 2 days prior to inoculation. The inoculation was performed by injection with a syringe of a bacterial suspension adjusted to 10^9 CFU ml^{-1} at the tip of growing shoots. Infected shoots (with necrosis $>$ 2 cm long from the inoculation point) were recorded 3 weeks later. Twenty shoots were inoculated per treatment and the experiment was repeated 2 times.

In the orchard, apple trees were sprayed repeatedly with ASM at 100 mg l^{-1} active ingredient (or water for the control) according to the following schedules: in experiment 1 (year 1), 2 successive sprays at 14 and 7 days before inoculation at pink and at tight cluster

respectively; in experiment 2 (year 2), 4 successive sprays at 7 and 2 days before inoculation at tight cluster and 1% bloom respectively, then at 7 and 14 days after inoculation at post bloom. Trees were inoculated at full bloom by spraying to run-off with a bacterial suspension of 10^8 CFU ml^{-1} (year 1) and 10^7 CFU ml^{-1} (year 2). The percent of infected clusters was determined by counting the total number of flower clusters 1 day after inoculation and infected clusters 30 days after inoculation. In each experiment and for each treatment (ASM or water), at least 120 clusters on each of five randomized trees were counted after inoculation.

Assays for enzymatic activities

These assays were only performed on apple seedlings grown in the greenhouse. In a first set of experiments, whole entire seedlings were sprayed at intervals with ASM (200 mg l^{-1} active ingredient) from 10 to 2 days prior to extraction. Young, nearly and fully expanded leaves (3 repeats of 2 leaves per treatment) were then sampled for enzyme analysis. In a second set of experiments, the youngest fully expanded leaf only of each seedling was carefully sprayed with ASM (200 mg l^{-1} active ingredient) (or water for control plants) while the upper nearly expanded leaf and the bud were carefully protected by wrapping them in an aluminium foil. The sprayed leaf and the adjacent upper leaf were separately sampled for independant enzyme investigation every two or three days after treatment for 17 days (3 repeats of 3 sprayed leaves and 3 repeats of 3 upper leaves per treatment). Each experiment was repeated at least twice.

After sampling, leaves were immediately homogenized with mortar and pestle in 50 mM cold sodium phosphate buffer pH 7.5 containing 1 mM polyethyleneglycol, 1 mM phenylmethylsulfonyl fluoride, 14 mM 2-mercaptoethanol, 8% polyvinypolypyrolidone and 0.01% Triton X-100 (10 µl of extraction buffer per mg of fresh weight). Homogenates were centrifuged at 17,000 × *g* for 20 min at 4 °C and supernatants were immediately assayed for enzyme activities.

Determination of peroxidase activity was based upon the oxidation of guaiacol in the presence of hydrogen peroxide (Chance and Maehly, 1955). Supernatants were diluted in 50 mM sodium phosphate buffer pH 7.5 (1 : 3). This diluted extract (50 µl) was added to a 1 ml reaction mixture that contained 50 mM sodium acetate buffer pH 5.5, 22 mM guaiacol and 20 mM H_2O_2. Oxidation of guaiacol to tetraguaiacol was monitored spectrophotometrically at 470 nm for 2 min at 30 °C. Peroxidase activity was expressed as nmol tetraguaiacol produced per min and per mg of proteins using a molar extinction coefficient of 26.6 mM^{-1} cm^{-1}.

β-1,3-Glucanase activity was determined according to the method of Wirth and Wolf (1992) with some modifications. Carboxymethyl-curdlan-Remazol Brilliant Blue (CM-curdlan-RBB, Loewe Biochemica, Otterfing, Germany) was used as substrate (Kalix and Buchenauer, 1995). CM-curdlan-RBB (100 µl of 4 mg ml^{-1} solution) and 1 M sodium acetate buffer pH 5 (100 µl) were first equilibrated in a water bath at 35 °C for 10 min. After the addition of crude supernatant (200 µl), the mixture was incubated at 35 °C for 1–2 h. The reaction was stopped by the addition of 2N HCl (100 µl) and the mixture was cooled on ice (10 min) and centrifuged at 10,000 × *g* for 10 min. Glucanase activity was calculated from the difference in absorbance at 600 nm between duplicate extracts incubated 1 and 2 h, respectively and expressed as the increase of absorbance per min and per mg of proteins.

Protein content in the extracts was determined according to the method of Bradford (1976) using the Coomassie® protein assay reagent (Pierce, Rockford, Illinois, USA).

Statistical analyses

Percentages of infection were transformed in arcsin for statistical analyses. Before each analysis, homogeneity of variances at $P = 0.05$ was checked by Bartlett's test. Data on seedlings (infections and enzyme activities) were analyzed by one-way analyses of variance and means were compared according to Duncan's multirange test. For data on blossom infections, the significance of difference was assessed by Student's *t* test.

Results

Protection of apple seedlings, scions and trees after ASM treatments

ASM provided significant control of fire blight whatever the plant material and the method of inoculation (spray on wounded leaves, shoot injection, flower spray) (Figure 1, Table 1). On seedlings, the different delays between treatments and inoculation (2–10 days)

and the two doses tested (100 or 200 mg l^{-1} active ingredient) gave similar results and the obtained protection was as effective as the protection obtained with streptomycin applied 4 h before inoculation, with an average level of protection of 75%. On scions, two repeated sprays of ASM before inoculation resulted in an average level of protection of 69%. On trees, a significant control of blossom blight of apple was recorded in the two experiments, with either two applications before or two applications before plus two applications after inoculation. In spite of a variable level of infection obtained in the water-sprayed check (due to different concentrations of inoculum and climatic conditions), the mean level of protection in both experiments approximated 50%.

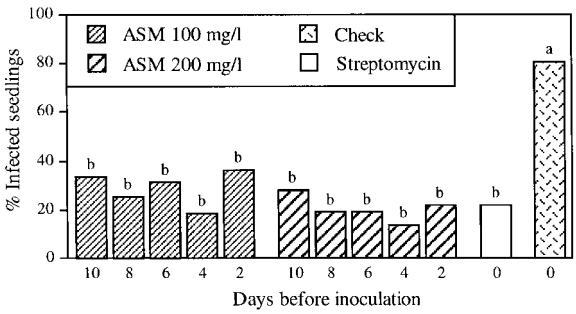

Figure 1. Efficacy of ASM (100 and 200 mg l^{-1} active ingredient) against fire blight on apple seedlings. ASM was applied once 10–2 days before wounding and inoculation with *E. amylovora* and compared with a standard streptomycin treatment and a check as described in Materials and methods. The data from two experiments with three replicates of 10 plants per treatment were pooled. Bars with the same letters represent values that are not significantly different ($P = 0.05$).

Correlation of protection with peroxidase and β-1,3-glucanase activities

Young leaves sampled from seedlings undergoing the same schedule of treatments as above were assayed for peroxidase and glucanase activities. Only the plants sprayed with the highest concentration of ASM (200 mg l^{-1}) were tested for these activities. Results show that higher activities of both enzymes peroxidases and β-1,3-glucanases were found in leaf tissues sampled on plants treated with ASM 2–10 days prior sampling when compared to the check or streptomycin-treated plants (Figure 2). Furthermore, the levels of activation increased progressively with the delay between ASM treatments and sampling.

Local and systemic activation of peroxidases and β-1,3-glucanases

Applications of ASM induced a progressive and significant increase of both enzymes in locally treated tissues and particularly for β-1,3-glucanases in upper untreated leaves when compared with control plants (Figure 3). For both enzymes, the level of induction was higher in treated leaves than in the upper untreated leaves. In treated leaves, this activation began earlier for β-1,3-glucanases (3 days after application of the chemical) than for peroxidases (5 days). However reactivity of treated leaves was slower for both enzymes in this experiment than in the experiment described above (Figure 2). This can be due to the fact that plants were younger on the day of chemical application (3–4 leaves versus 8–10 leaves) and that they received a smaller amount of the chemical (one leaf per plant only was sprayed versus the whole plant). Therefore

Table 1. Efficacy of ASM (100 mg l^{-1} active ingredient) against fire blight on Golden Delicious scions in the greenhouse and on Golden Delicious trees in the orchard

Treatment	Scions[1]		Trees[2]	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
ASM	20	30	38 a	14 x
Water	80	80	65 b	30 y

[1]Percentages of infected shoots. ASM (or water) was applied twice before inoculation as described in Materials and methods; 20 shoots inoculated per treatment and per experiment.

[2]Percentages of infected clusters. ASM was applied twice before inoculation, and twice after (experiment 2 only) as described in Materials and methods. Means of five replicates of at least 120 clusters each on five randomized trees; a–b, x–y, significant differences in experiments 1 ($P = 0.05$) and 2 ($P = 0.01$), respectively.

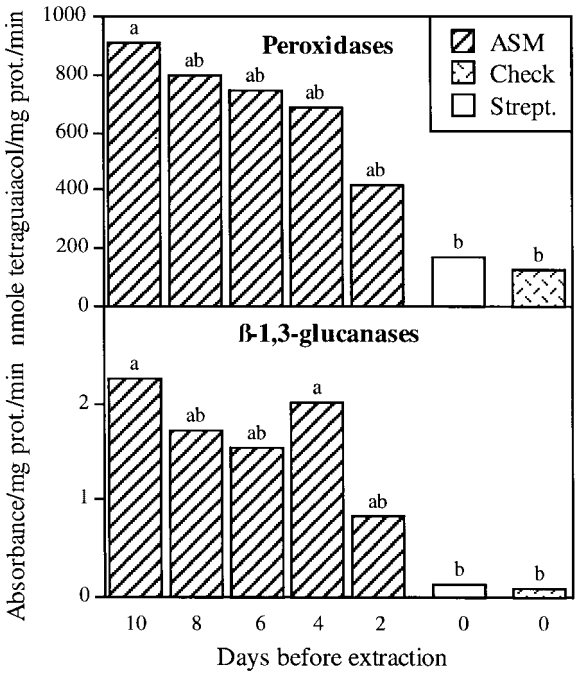

Figure 2. Induction of peroxidases and β-1,3-glucanases in young leaves of apple seedlings previously treated with ASM (200 mg l^{-1} active ingredient). ASM was applied once 10–2 days before enzyme extraction and compared with a standard streptomycin treatment and a check as described in Materials and methods. Results are from one representative experiment. The experiment was performed three times. Bars are the means of three separate extractions. Bars with the same letters represent values that are not significantly different ($P = 0.05$).

the chemical induction may have been less intense. In untreated leaves, the delay for activation was longer, at least 10 days for both enzymes.

Discussion

ASM provided a significant control of fire blight after leaf infection of apple seedlings and shoot inoculation of apple scions in the greenhouse and after blossom infection of field-grown apple trees. Using greenhouse plant material, this protection was clearly correlated with the activation of defense mechanisms as indicated by the induction of two defense markers, peroxidases and β-1,3-glucanases, in treated leaves as well as in untreated upper leaves. Because we did not compare with a biological activator of SAR, we cannot state that ASM induces SAR in this plant species. However since this chemical does not demonstrate any bacteriostatic or bactericidal effect *in vitro* (data not shown) and systemically activates two well-known defense-related enzymes, these data provide first evidence that ASM triggers systemic resistance in apple.

Timing of triggering local and systemic protection is not easy to examine in the case of fire blight. Apart from blossoms, only young vegetative parts are receptive to the bacterial infection because tissues quickly becomes physiologically resistant as they age. Concerning local protection, it is then not possible to dissociate a chemical induction of resistance from an aging effect on the progress of infection. And concerning systemic protection, it is not possible to keep a constant distance between treated leaves and inoculated leaves. That is why we first established a relationship between a global protection of seedlings previously sprayed with ASM and concurrent enzyme activation in leaves equivalent to those inoculated. Then in separate experiments, we demonstrated the local and systemic activation of these enzymes.

Biochemical markers of SAR have not been previously reported in apple. We arbitrarily choose two enzymes whose role in SAR has been previously demonstrated in a number of plant species (essentially annual plants). Peroxidases participate into cell-wall reinforcement. They are involved in the final steps of lignin biosynthesis and in the cross-linking of cell-wall proteins (Kombrink and Somssich, 1995). They are usually related to local defense responses but they have been associated with systemic resistance in several plant species (cucumber (Hammerschmidt et al., 1982), potato (Chai and Doke, 1987), tobacco (Ye et al., 1990)). In apple, the present results show a significant local accumulation of these enzymes and a persistant tendency to their systemic accumulation. They could increase the resistance against *E. amylovora* by accelerating the rigidification of the cell-walls of young tissues similar to the resistance associated with normal aging. The bacteria establish and multiply best in intercellular spaces of expanding parenchyma cells and cell-wall fortification could limit their progression. β-1,3-Glucanases are PR proteins in diverse dicots and monocots (Shewry and Lucas, 1997). They demonstrate mainly antifungal activity by hydrolyzing the cell-walls of many phytopathogenic fungi. Therefore, in contrast to peroxidases they are probably not directly involved in resistance against *E. amylovora*, but their significant local and systemic accumulation is an indication of an induction of overall resistance to pathogen attack.

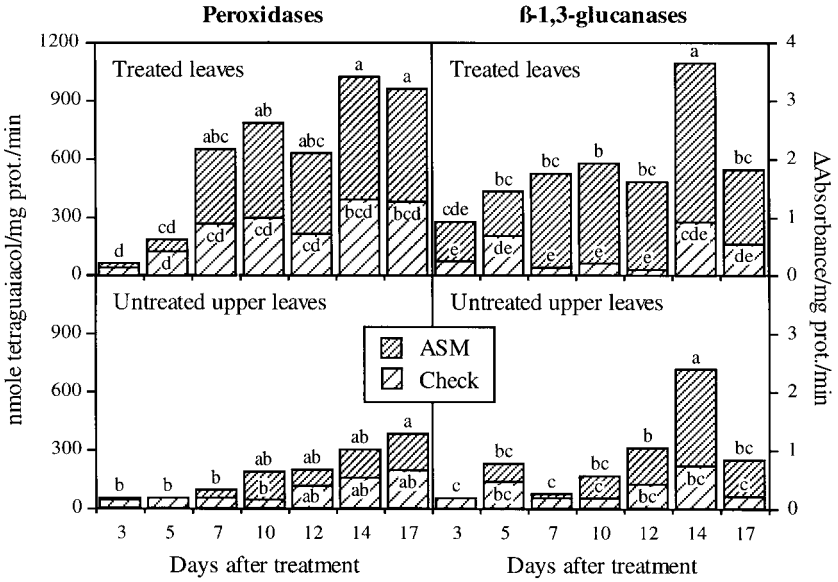

Figure 3. Time-course of peroxidase and glucanase inductions in leaves treated with ASM (200 mg l^{-1} active ingredient) and water (check), and in upper untreated leaves. Results are from one representative experiment. The experiment was performed twice. Bars are the means of three separate extractions. Bars with the same letters represent values that are not significantly different ($P = 0.05$).

From our data, the relationship between protection against infection and activation of defense markers in treated leaves is clear. Protection against fire blight was generally obtained when ASM was applied 2 days before inoculation, and enzyme analyses in identically treated leaf tissues indicated that bacteria would be exposed to tissues initiating an increase in defense reactions. Infection of tissues is not immediate after inoculation because the pathogen may require several days to become established in infection sites. This provides additional time for the plant to respond to the inducing compound and reach higher levels of defense induction. In addition there are most likely other defense reactions and compounds that are also probably involved in the observed protection. They could be more rapidly induced than the markers chosen in this study, and could be more effective against *E. amylovora*.

Induction of β-1,3-glucanases (and to some extent of peroxidases) in untreated upper leaves demonstrates the systemic nature of ASM action in apple. In addition the sustained induction of both enzymes locally and systemically for 17 days, as well as the protection obtained on plants inoculated as late as 10 days after treatment (young inoculated leaves were most probably not present at the time of treatment) suggest that ASM provides a sustained protection in apple. These observations apply to actively growing seedlings and whether the same induction occurs in the field on adult plant material remains to be determined. However it should be emphasized that systemic induction and long-term protection are two beneficial responses for practical application of ASM that would improve fire blight control on mature trees. Two growth stages of trees are especially prone to infection: blossom period and shoot growth phase (Billing, 1980a). Application of the chemical inducer on flower buds resulting in the protection of open flowers from infection would be an ideal strategy for the control of blossom blight, usually the most dangerous phase of this disease. It would not be so critical then to be absolutely accurate in the timing of application, as it is the case with bactericide compounds (Billing, 1980b; Thomson et al., 1982; Steiner, 1990). Similarly, treatment of actively growing shoots to protect succulent young tissues (another crucial phase in infection) would be a great improvement for shoot blight control.

All genera of *Maloideae* are potential hosts for fire blight, some of them of equal economic importance as apple. Because of the unique mode of action of ASM, it can be expected that its overall effect against a specific pathogen will more or less depend upon the plant which is treated. It is likely that each species and perhaps

even each cultivar may respond differently to chemical induction. This chemical remains to be tested on pear or on other host plants such as ornamentals (*Cotoneaster, Pyracantha*). However, further data will be needed on the stability of protection, dose response and an appropriate spray schedule before the practical application of ASM in the orchard. The demonstration of markers of defense induction in apple (and in other host plants of fire blight) could provide a valuable tool for further studies for field trials. According to our results, peroxidases and β-1,3-glucanases can be recommended as routine markers of defense reactions in apple.

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