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Induction of resistance to fire blight in apple by acibenzolar-S-methyl and DL-3-aminobutyric acid

Resistenzinduktion gegenüber Feuerbrand an Apfel durch Acibenzolar-S-methyl und DL-3-Aminobuttersäure

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Summary

Fire blight disease caused by *Erwinia amylovora* is one of the most important bacterial diseases of apple and pear worldwide. DL-3-aminobutyric acid (BABA; 1.0 mg ml^{-1}) and acibenzolar-S-methyl (ASM; 0.1 mg ml^{-1}) were applied as foliar treatments on apple seedlings at various days before inoculation (dbi) either alone or in combination at different time sequences. ASM was slightly more effective than BABA in controlling fire blight disease expressed as browning discolouration index (BDI) and stem bending index (SBI). Combined treatments in sequence with BABA/ASM drastically reduced disease expression (BDI 98.1%, SBI 97.8%) for a long period and proved to be more effective than the other treatments. The highest degrees of induced resistance were obtained when BABA and ASM were applied in sequence 4 and 2 dbi or 6 and 4 dbi, respectively. The resistance inducing effects gradually decreased when the period of time between treatment and inoculation increased. Peroxidase (PO) activities increased more markedly in inoculated leaf tissues treated with ASM and BABA/ASM than in BABA-treated plants and in inoculated control tissues. Inoculated or not inoculated apple seedlings treated with BABA alone or combined with ASM (BABA/ASM) revealed the highest levels of both total and free salicylic acid (SA) in leaf tissues. Moreover, in leaf tissue treated with the combination BABA/ASM the lowest number of bacterial cells was determined.

Key words: acibenzolar-S-methyl (ASM), DL-3-aminobutyric acid (BABA), fire blight, peroxidase (PO), salicylic acid (SA)

Zusammenfassung

Feuerbrand, hervorgerufen durch *Erwinia amylovora*, gehört weltweit zu den bedeutendsten Bakterienkrankheiten an Apfel und Birne. Apfelsämlinge wurden im Sprühverfahren mit DL-3-Aminobuttersäure (BABA; 1,0 mg ml^{-1}) und Acibenzolar-S-methyl (ASM; 0,1 mg ml^{-1}) an unterschiedlichen Tagen vor Inokulation (dbi) entweder allein oder in Kombination in verschiedener zeitlicher Sequenz behandelt. Hinsichtlich der Unterdrückung der Feuerbrandsymptome, die als Blattverbräunungsindex (BDI) und Stängelkrümmungsindex (SBI) ausgewertet wurden, erwies sich ASM als etwas stärker wirksam als BABA. Kombinierte Behandlung mit BABA/ASM in Sequenz verminderte drastisch den Krankheitsbefall (BDI 98,1%, SBI 97,8%) während einer langen Zeitspanne und erwies sich als wirksamer als die übrigen Behandlungen. Die stärkste Resistenz-induzierende Wirkung wurde erzielt, wenn BABA und ASM in Sequenz 4 und 2 dbi oder 6 und 4 dbi angewendet wurden. Die Resistenz-induzierende Wirkung verminderte sich bei zunehmender Zeitspanne zwischen Behandlung und Inokulation. Peroxidase (PO)-Aktivitäten nahmen in inokulierten Blattgeweben, die mit ASM und BABA/ASM behandelt worden waren, stärker zu als in BABA-behandelten Pflanzen und inokulierten Kontrollgeweben. Inokulierte oder nicht inokulierte Apfelsämlinge, die mit BABA allein oder in Kombination mit BABA/ASM behandelt worden waren, zeigten in den Blattgeweben die höchsten Gehalte an gesamter und freier Salicylsäure (SA). Darüber hinaus wurde in BABA/ASM-behandelten Blättern die niedrigste Zahl an Bakterienzellen nachgewiesen.

Stichwörter: Acibenzolar-S-methyl (ASM), DL-3-Aminobuttersäure (BABA), Feuerbrand, Peroxidase (PO), Salicylsäure (SA)

1 Introduction

Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al., is a classic disease of pome fruits. The disease is very destructive on apple and pear (Bereswill et al. 1998), and it is one of the most difficult diseases to control (Norelli et al. 2003). Copper compounds have low effectiveness in disease control, and spray applications during flowering often cause undesirable phytotoxic effects on fruit finish and reduce fruit quality (Thomson et al. 1999). Streptomycin is considered the most effective bactericide against this disease, causing no real phytotoxic problems at recommended rates. Its application is debated controversially, and in many countries the use of this compound is not allowed due to the risk of inducing antibiotic resistance in human and veterinary microbial pathogens (Halbwirth et al. 2002). After many years of application of streptomycin, resistant isolates first emerged in California, later in other states of the USA. Streptomycin-resistant strains of *E. amylovora* have recently also been isolated in Israel (Manulis et al. 1999), Egypt (El-Goorani et al. 1989), New Zealand and in several other countries (Vanneste 2000). Research for new substances against *E. amylovora* over the last 15 years has resulted in certain compounds with activity against fire blight. Some promising compounds, for instance, are inducers of disease resistance. Induced resistance (IR) as a general phenomenon in plants has been studied in many host plant-pathogen interactions in recent years. Systemic acquired resistance (SAR) can also be induced by exogenous application of some synthetic compounds (Siegrist et al. 2000; Zimmerli et al. 2001; Baysal et al. 2002). Resistance expressed is generally effective against a broad range of pathogens and is associated with the production of PR proteins. (Hammerschmidt 1999).

Acibenzolar-S-methyl (ASM), known under the commercial name Bion®, is one of the non-toxic synthetic resistance inducers used against plant pathogens (Kunz et al. 1997). The compound is known to elicit SAR against fungal and bacterial diseases of several plants including tobacco, cucumber (Lawton et al. 1996) and apple (Zeller and Zeller 1999; Baysal et al. 2002). DL-3-aminobutyric acid (BABA) is a non-protein amino acid which induces resistance against a large number of plant pathogens such as viruses, bacteria, fungi and nematodes (Zimmerli et al. 2001; Cohen 2002). Synergistic interactions between BABA and ASM have been reported in tobacco plants against *Peronospora tabacina* (Reuveni et al. 2001).

The objective of this study was to compare the resistance-inducing effects of BABA with ASM against fire blight in apple seedlings when applied individually. So far, no research

has been reported on activation of resistance by BABA on this disease. In addition, the potential of enhanced fire blight control was tested when both compounds were applied in sequence. Finally, biochemical studies on the mode of induction of resistance were performed.

2 Materials and methods

2.1 Apple seedlings

Apple (*Malus domestica* Borkh.) seeds of cv. Golden Delicious were immersed 3 days in tap water at 4°C in a refrigerator; the water was exchanged daily. Then, the seeds were immersed in 0.1% active ingredient (a.i.) of Euparen® (Bayer A. G.; 50% dichlofluanid) suspension for 5 min to prevent fungal growth. Seeds were then transferred to sterile glass Petri dishes (15 cm in diameter) on filter paper, 30 ml of sterile water were added to each Petri dish and the seeds were incubated at 4°C for 4–6 weeks to break seed dormancy. The germinated seeds were sown individually in pots (13 cm in diam.) containing a mixture of peat, sand and vermiculite (3:1:1, v/v/v). Seedlings were cultivated in the greenhouse at temperatures between 17 and 22°C under natural photoperiods during the growing season and supplemented with artificial light during fall and winter. Seedlings were fertilized with 100 ml pot^{-1} of 0.15% Wuxal-Super® liquid fertilizer (NPK: 8-8-6) each 10 days. Six weeks-old apple seedlings (about 8-10 leaves) were used for experiments.

2.2 Inoculum preparation

The isolate of *Erwinia amylovora* (Er18) (culture collection of the Institute of Phytomedicine, University of Hohenheim) was stored in sterile distilled water at 4°C. For cultivation, a diluted bacterial suspension was transferred onto King's B agar medium in Petri dishes (King et al. 1954) and cells were incubated at 27 ± 2°C for 24 h. A single colony of the isolate was selected and grown in a 250 ml Erlenmeyer flask containing 100 ml of nutrient sucrose broth (NSB) and incubated at 27 ± 2°C for 24 h on a rotary shaker at 175 rpm. The culture was used as inoculum after adjusting cell density with sterile water to 1 x 10^8 cfu ml^{-1} using a spectrophotometer at a wavelength of 600 nm.

2.3 Inoculation and evaluation of disease

Inoculation was carried out by dipping sterilised scissors in bacterial suspension for 20 sec before cutting the tips (about 1.5 cm from the tip) of two young leaves (beneath the two apical ones) of apple seedlings as mentioned by Norelli and Gilpatrick (1982) with some modifications. In case of control seedlings, sterile distilled water was used instead of bacterial suspension. Inoculated seedlings were placed in a dew chamber at 100% relative humidity (RH) and 24 ± 2°C for 24 h and subsequently transferred to the greenhouse. Ten days after inoculation, disease indexes of infected seedlings were calculated using the following scales.

Scale 1: Browning discoloration index (BDI): Disease severity (%) of fire blight on apple seedlings was evaluated using a rating system from 0 − 6 according to the modified scale described by Schilli (1986): 0 = no leaf symptoms; 1 = 25% or less; 2 = 25 − 50%; 3 = 50 − 75%; 4 = > 75% brown discoloration from the edge of cutting to the midrib; 5 = > 75% brown discoloration from the edge of cutting to the midrib and/or the petiole turns brown; 6 = the petiole is brown or black and releasing bacterial exudates "ooze".

BDI (in %) was calculated as follows:

$$\text{BDI (\%)} = \frac{\Sigma\,(\text{number of leaves} \times \text{class of symptom})}{\text{number of leaves evaluated (2)} \times \text{maximum score (6)}} \times 100$$

Scale 2: Bending of seedlings was rated by the following scale described by Beer et al. (1983): 0 = seedlings and leaves fully turgid; 1 = leaf lamina flaccid, stem turgid; 2 = stem bent 0-30 degrees from vertical; 3 = stem bent 30-60 degrees from vertical; 4 = stem bent 60-90 degrees from vertical; 5 = stem bent > 90 degrees.

$$\text{Stem bending index (SBI) \%} = \frac{\text{class of symptom}}{\text{maximum possible score (5)}} \times 100$$

2.4 Evaluation of plant resistance inducers on inhibition of Erwinia amylovora *growth* in vitro

The toxic effects of ASM and BABA were tested against *E. amylovora* using the impregnated filter paper disk method (Sholberg et al. 2001). Four replicates were used for each treatment. After incubation the inhibition zone around each disk was measured and the area of inhibition was expressed in mm^2.

2.5 Greenhouse experiments

To study the effect of different treatments of BABA and ASM on induction of resistance, apple seedlings were sprayed with BABA (1 mg ml^{-1}) 4 dbi or ASM (0.1 mg ml^{-1}) 2 dbi singly and both compounds were sprayed simultaneously (combination). In addition, the inducers of resistance were applied in sequence, whereby BABA or ASM was sprayed first 4 dbi and 2 days later the other compound (2 dbi) (BABA/ASM or ASM/BABA). Control seedlings were sprayed with sterile distilled water. Four days after the first treatment apple seedlings were inoculated with cell suspension of *E. amylovora*. In this experiment 10 apple seedlings were used as replicates per treatment and experiments were repeated three times.

In order to determine the time interval for optimal expression of acquired resistance, apple seedlings were sprayed with BABA 14, 12, 10, 8, 6 and 4 dbi, and 2 days later with ASM. Control seedlings were sprayed with water. Four apple seedlings were used for each variant. This experiment was repeated twice.

To determine the effect of BABA/ASM treatment on growth of *E. amylovora* in leaf tissues, apple seedlings were treated with BABA (1 mg ml^{-1}) 4 dbi and ASM (0.1 mg ml^{-1}) 2 dbi. Ten days after inoculation the BDI and SBI were evaluated. Then, one gram fresh weight of leaves of apple seedlings from relevant treatments was used from each treatment. Samples were homogenized in a sterile mortar and pestle with 5 ml of 0.1 M potassium phosphate buffer (pH 7.0). Leaf homogenates were diluted (from 10^{-1} to 10^{-8}). Then 100 µl of each dilution were transferred on King's B agar medium in Petri plates. The plates were incubated at 27 ± 2°C for 24 h and the number of bacterial colonies was counted.

2.6 Physiological studies

For quantification of free and total salicylic acid (SA) and determination of peroxidase (PO) activity in leaf tissues apple seedlings were sprayed with aqueous solutions of BABA (1 mg ml^{-1}) 4dbi and ASM (0.1 mg ml^{-1}) 2 dbi alone or in combination, as well as in sequence (4 and 2 dbi as described before). For control, apple seedlings were sprayed with sterile distilled water. One half of the treated apple seedlings was inoculated with *E. amylovora* 2 days after the last treatment. The two leaves beneath the two apical ones of each treatment were sampled 0, 2, 4, 6, and 8 days after the last treatment to determine SA contents and PO activities.

Free and total SA were extracted from leaves of apple seedlings according to the method of Malamy and Klessig (1992). For determination of PO activity 1 g of leaf tissue was

immersed in liquid nitrogen in a prechilled mortar and pestle, and then homogenized in 10 ml sodium phosphate buffer (10 mM at pH 7). The homogenates were centrifuged at 15,000 rpm and 4°C for 30 min. The supernatants were stored at −20°C or immediately used for peroxidase assays. Determination of PO activity was based upon the oxidation of guaiacol in the presence of hydrogen peroxide (HAMMERSCHMIDT et al. 1982). One-hundred µl of the homogenate were incubated with 500 µl 0.1 M Na-acetate buffer (pH 5.2), 200 µl 1% guaiacol and 200 µl 1% H_2O_2 at room temperature for 10 min. PO was measured spectrophotometrically (Pharmacia LKB Biochrom 4060) at 470 nm (YE et al. 1990). A mixture of Na-acetate buffer, guaiacol and H_2O_2 was used as a blank. The standard curve of PO was prepared using peroxidase enzyme from horseradish (pure; 225 U mg^{-1}) at 0.1, 0.3, 0.5, 1, 2 and 2.5 µg ml^{-1}, dissolved in 10 mM sodium phosphate buffer (pH 7). The protein content in the sample extracts (1 g) was determined according to the method of BRADFORD (1976) as follows:

$$\text{Enzyme content } (\mu g\ mg^{-1}\ \text{protein}) = \frac{\text{Enzyme content in one gram of leaves } (\mu g\ mg^{-1})}{\text{Protein content in one gram of leaves } (\mu g\ mg^{-1})} \times 100$$

2.7 *Statistical data analysis*

The data were analyzed by SPSS (version 10 for Windows) using the one-way analysis of variance (ANOVA). Differences between treatment means were determined using Tukey`s test ($P = 0.05$). Different letters indicate significant differences among treatments or means indicated with an asterisk (*) differ significantly from the infected control treatment. Means of standard deviation for five or 10 replicates per treatment are shown.

3 Results

3.1 *Pathogenicity of* Erwinia amylovora *on apple seedlings*

The isolate Er18 was pathogenic on apple seedlings and produced typical symptoms, causing a browning discolouration index (BDI) of 95.8% and a stem bending index (SBI) of 95%.

3.2 *Effect of plant resistance inducers on* Erwinia amylovora *in vitro*

The effect of resistance inducers on growth of *E. amylovora* isolate Er18 was measured as inhibition zone area in mm^2. BABA (0.5 and 1.0 mg ml^{-1}) and ASM (0.1 and 0.2 mg ml^{-1}) did not inhibit bacterial growth.

3.3 *Effect of single foliar treatments with DL-3-aminobutyric acid (BABA) and acibenzolar-S-methyl (ASM) as well as combinations of both compounds on fire blight*

Compared to untreated apple seedlings, single spray applications with BABA 4 dbi or ASM 2 dbi reduced BDI by 44.1%, 51.9% and SBI by 46.7%, 66.7%, respectively (Fig. 1). When BABA and ASM were applied in sequence 4 and 2 dbi disease expression was drastically reduced (BDI 98.1%, SBI 97.8%). Moreover, BABA/ASM-induced resistance in apple seedlings was long lasting and disease symptoms did not appear for more than 2 months (data not shown). Also when apple seedlings were sprayed with ASM 4 dbi and then with BABA 2 dbi both disease symptoms were significantly reduced (BDI 94.1%, SBI 95.5%). However, when both compounds were applied simultaneously 2 dbi (combination) disease severity of fire blight was reduced less effectively compared to the treatments in sequence.

3.4 *Effect of BABA, ASM on fire blight and bacterial number in apple seedlings*

Spraying of apple seedlings with BABA 4 dbi and ASM 2 dbi (BABA/ASM) significantly decreased the disease indexes of fire blight compared to the inoculated control. Also the bacterial populations of *E. amylovora* were reduced in BABA/ASM-treated apple seedlings (2.7×10^5) compared to the untreated inoculated seedlings (7.2×10^7 cfu g^{-1}) (Table 1).

3.5 *Determining the optimal time interval between treatment with BABA/ASM and inoculation for fire blight control*

The time course studies of expression of induced resistance following application of BABA (14, 12, 10, 8, 6 and 4 dbi) and

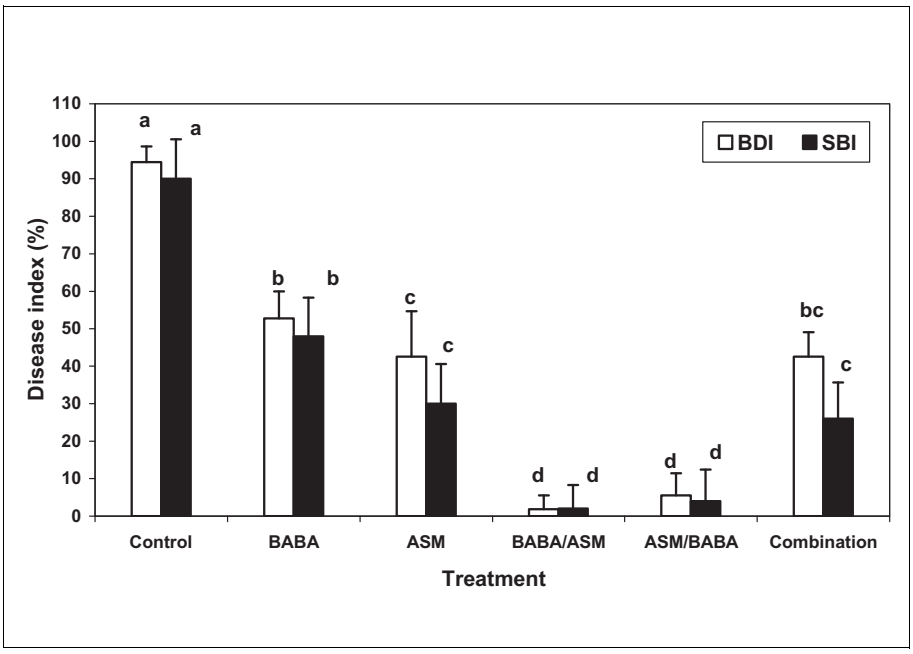


Fig. 1: Effects of single foliar treatments of apple seedlings with DL-3-aminobutyric acid (BABA; 1.0 mg ml^{-1}) 4 dbi, acibenzolar-S-methyl (ASM; 0.1 mg ml^{-1}) 2 dbi and applications of both compounds in sequence 4 dbi and 2 dbi, whereby BABA was sprayed before or after ASM. BABA and ASM were also applied simultaneously 2 dbi (combination). Control seedlings were sprayed with sterile distilled water. Apple seedlings were inoculated with *Erwinia amylovora* (1×10^8 cfu ml^{-1}), and 10 days after inoculation both browning discolouration index (BDI) and stem bending index (SBI) were evaluated. Different letters indicate significant differences among treatments according to least significant difference test ($P = 0.05$). Means of standard deviation for ten seedlings per treatment are shown.

Table 1: Effect of spraying apple seedlings with DL-3-aminobutyric acid (BABA) (1.0 mg ml^{-1}) 4 dbi and acibenzolar-S-methyl (ASM) (0.1 ml^{-1})) 2 dbi (BABA/ASM) on fire blight disease indexes and number of *E. amylovora* cfu per g infected leaf tissue. Data were determined 10 days after inoculation. Different letters indicate significant differences among treatments according to least significant difference test (P= 0.05).

Treatments	Disease index		Bacterial number (cfu g^{-1} leaf tissue)
	BDI *	SBI **	
Infected control	93.75 a	85 a	7.2 x 10^7
BABA/ASM	2.08 b	0.00 b	2.7 x 10^5
Non-infected control	0.00 b	0.00 b	0

* Browning discolouration index (BDI)
** Stem bending index (SBI)

ASM (12, 10, 8, 6, 4 and 2 dbi) in apple seedlings against fire blight, indicated that the highest degree of resistance was obtained when BABA was applied 4 dbi and ASM 2 dbi (Fig. 2). BDI was reduced by 97.8% and SBI by 100%. Treatment 6 and 4 dbi with BABA and ASM, respectively, also resulted in drastically diminished BDI (95.6%) and SBI (100%). Resistance inducing effects gradually decreased with increasing time between treatment and inoculation.

3.6 Activation of peroxidase (PO) in apple seedlings

Applications of ASM or BABA alone or in sequence (BABA/ASM) induced a significant increase of PO activity in treated not-inoculated leaf tissues at all sampling times compared with control tissues (Fig. 3A). The highest levels of PO were determined 2 and 4 days after ASM treatment and enzyme levels decreased slightly at the two later sampling times. After BABA treatment the PO levels increased more slowly until 6 days after treatment (dat), and at the later sampling times the enzyme activity decreased. BABA/ASM induced 4/2 dat a high level of PO activity in leaf tissues, afterwards PO levels decreased. Generally, higher activities of PO were determined in leaf tissues treated with either ASM alone (2 dat) or combined with BABA (BABA/ASM) 4/2 dat.

Leaves of inoculated apple seedlings of all treatments showed higher PO levels than the corresponding not inoculated leaf tissues. In untreated and inoculated leaves, PO levels slightly increased 2 dai and remained at the same level during the experimental period (Fig. 3B). The level of PO was already slightly increased in leaves treated with BABA (4 dat) immediately before inoculation and after inoculation enzyme activity slowly progressed. In leaves of apple seedlings with ASM alone (2 dat, immediately before inoculation), the PO activities were high and further increased at 4 dat (2 dai). At the following two sampling times the PO activity remained at the same level. Double treatment with BABA and ASM (BABA/ASM) also induced a rapid increase of PO levels in apple leaves comparable to those determined in ASM-treated leaves. In summary, PO accumulated more markedly in inoculated leaf tissues treated with ASM and BABA/ASM than in BABA-treated plants. Infection with *E. amylovora* only slightly induced PO activity in leaves of apple seedlings.

3.7 Accumulation of salicylic acid (SA) in apple seedlings

The lowest amount of free SA was found in leaves of not-inoculated control seedlings and in leaves treated with ASM singly (Fig. 4A). In apple seedlings treated with BABA alone a high accumulation of free SA was induced already 4 dat compared with the control. Six, 8 and 10 dat with BABA similar contents of free SA were determined. High free SA contents were also determined in apple seedlings treated with BABA/ASM during the experimental period. However, when plants were treated with ASM/BABA (4/2 dat), the free SA levels were significantly lower compared to leaves treated with BABA alone or BABA/ASM. In leaves of apple seedlings treated simultaneously with BABA and ASM (combination), the content of free SA slightly increased 2 and 4 dat and decreased 6 and 8 dat.

Determination of total SA contents (Fig. 4B) resulted in about 10 times higher levels than those of free SA. Low total SA contents were determined at all sampling times in leaves of the control, and in leaves treated with ASM no appreciable SA accumulation was observed. On the other hand, in plants treated with BABA, increasing total SA contents were analyzed 4, 6, 8 and 10 dat. In plants treated with BABA/ASM or ASM/BABA total SA contents were somewhat lower compared

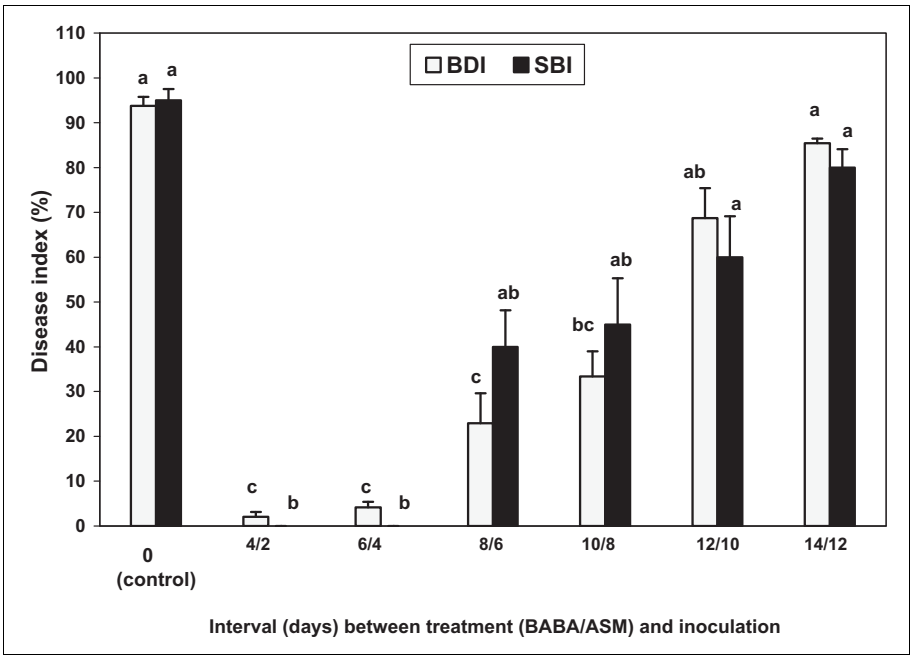


Fig. 2: Effect of time intervals between application of BABA/ASM and inoculation with *E. amylovora* (1 x 10^8 cfu ml^{-1}) on fire blight. Apple seedlings were sprayed with BABA (1 mg ml^{-1}; 14, 12, 10, 8, 6 and 4 dbi), then two days later with ASM (0.1 mg ml^{-1}). Disease avaluation and statistics see Fig. 1.

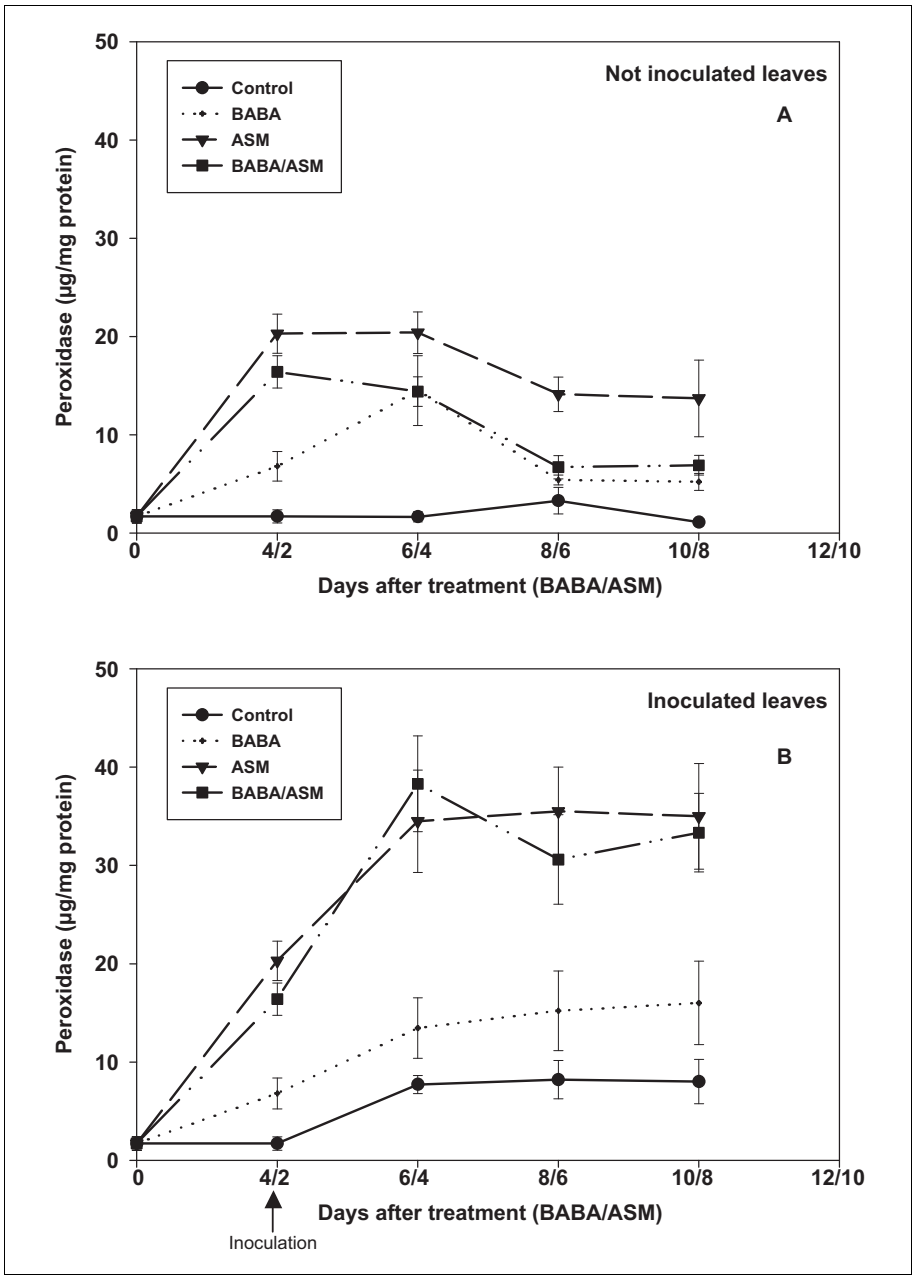


Fig. 3: Induction of peroxidase (PO; µg/mg protein) activity in not inoculated (Fig. 3A) and *Erwinia amylovora*-inoculated (Fig. 3B) leaves of apple seedlings previously treated with BABA (1 mg ml^{-1}) and ASM (0.1 mg ml^{-1}). Single treatment with BABA was 10, 8, 6, and 4 days and of ASM 8, 6, 4, and 2 days before enzyme extraction. Both BABA and ASM were also applied in sequence, whereby ASM was given 2 days later than BABA (BABA/ASM). Means of standard deviation for five seedlings per treatment are shown.

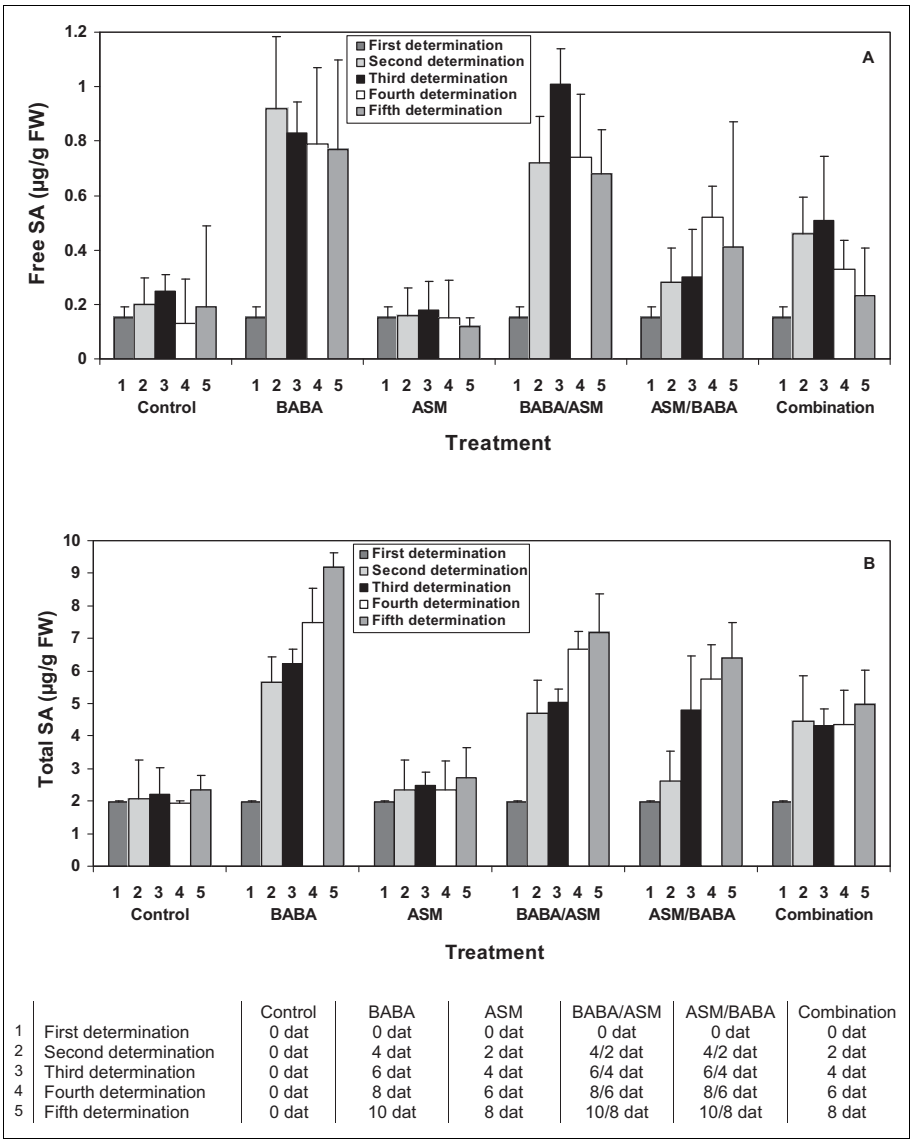
to the corresponding sampling times of BABA-treated leaves. The total SA concentration in leaves treated simultaneously with BABA and ASM (combination) did not increase with extending sampling time. Generally, apple seedlings treated with BABA alone or combined with ASM (BABA/ASM) revealed the highest levels of both total and free SA contents in leaf tissues.

In not inoculated control leaves free SA contents were very low (Fig. 5A). Treatment of seedlings with BABA induced a high accumulation of free SA already before inoculation with *E. amylovora* (4 dat). At the following two sampling times free SA contents further increased. On the other hand, ASM did not induce accumulation of free SA in inoculated apple leaves. Treatment with BABA and ASM in sequence (BABA/ASM) also strongly increased free SA contents in leaves 2 and 4 dai. In leaves treated with ASM/BABA free SA concentrations were significantly lower than in leaf tissue treated with BABA/ASM. In the simultaneous treatment (combination) the levels of free SA in leaf tissues were significantly lower than in the BABA/ASM treatment. In conclusion, the highest levels of free SA were found in leaves of seedlings treated with BABA alone and BABA/ASM.

In general, total SA levels in leaves of apple seedlings were approximately 8-9 times higher than those of free SA contents (Fig. 5B). In inoculated control leaves total SA contents were only slightly higher than in not infected control tissues. In BABA-treated leaves total SA content was already high before inoculation and at 4 dai the highest value of SA was determined. ASM application did not induce an increase in total SA contents in inoculated leaf tissues. At the corresponding sampling times, the contents of total SA were lower in leaves treated with BABA/ASM or ASM/BABA than in tissues treated exclusively with BABA. In the combined treatment total SA contents did not increase with time after inoculation. In summary, apple seedlings treated with BABA alone or combined with ASM (BABA/ASM) showed the highest free and total SA contents after inoculation.

4 Discussion

The inducers of resistance BABA and ASM did not exhibit direct toxicity to *E. amylovora*, as was expected from reports



		Control	BABA	ASM	BABA/ASM	ASM/BABA	Combination
1	First determination	0 dat	0 dat	0 dat	0 dat	0 dat	0 dat
2	Second determination	0 dat	4 dat	2 dat	4/2 dat	4/2 dat	2 dat
3	Third determination	0 dat	6 dat	4 dat	6/4 dat	6/4 dat	4 dat
4	Fourth determination	0 dat	8 dat	6 dat	8/6 dat	8/6 dat	6 dat
5	Fifth determination	0 dat	10 dat	8 dat	10/8 dat	10/8 dat	8 dat

Fig. 4: Accumulation of free (Fig. 4A) and total (Fig. 4B) salicylic acid (SA) (µg g^{-1} FW) in not inoculated leaves of apple seedlings after treatment with BABA (1.0 mg ml^{-1}) and ASM (0.1 mg ml^{-1}). Apple seedlings were treated with BABA and ASM singly, in sequence as well as in combination. BABA and ASM were also applied simultaneously (combination). Means of standard deviation for five seedlings per treatment are shown.

on their activity against fungi and bacteria (Oostendorp et al. 2001; Cohen 2002). Both compounds act as activators of systemic acquired resistance (SAR) against a similar spectrum of pathogens.

The studies have been performed with the apple cultivar 'Golden Delicious' which consists of various mutants (e.g. colour, rasseting).

Spray treatments of apple seedlings with BABA (4 dbi) and ASM (2 dbi) in sequence drastically reduced fire blight symptoms, the browning discolouration index (BDI) and stem bending index (SBI), while single treatments with BABA (4 dbi) and ASM (2 dbi) diminished both disease symptoms less effectively. Furthermore, BABA/ASM treatment induced long lasting resistance in apple seedlings; for more than two months no disease symptoms developed. When ASM was applied first (4 dbi) followed by BABA (2 dbi) disease symptoms were also drastically reduced. The marked effects of induced resistance following treatment with ASM are consistent with several reports not only in apple but also in other plants (Norelli et al. 2003).

The broad spectrum of protective effectiveness of BABA against numerous plant diseases has been well documented. Jakab et al. (2001) and Cohen (2002) reported that BABA operates via a variety of defense mechanisms including physical barriers and biochemical changes. Activation of resistance has been reported to depend on SA accumulation and lesion formation (Siegrist et al. 2000). To our knowledge, defense responses in apple seedlings induced by foliar applications of BABA have not been reported. Our results revealed markedly enhanced free and total SA levels after treatment with BABA. It may be suggested that BABA induced resistance in apple seedlings also through activation of a signalling pathway that depended on SA accumulation. Some reports indicated that BABA induced synthesis and accumulation of pathogenesis-related (PR) proteins in tomato plants including PR-1, chitinase, β-1,3-glucanase (Cohen et al. 1994) and AP24 (Jeun and Buchenauer 2001). Studies also revealed that BABA treatments induced production of phenolics, peroxidases, callose, lignin and other defense reactions depending on the host-pathogen system. On the other hand, it has been shown by many studies in numerous crops that activation of SAR by ASM is SA-independent. ASM is translocated both in the xylem and phloem of plants and acts in the signal pathway of SAR induction at or downstream of the SA site of action (Sticher et al. 1997; Oostendorp et al. 2001). ASM induces the expression of PR protein genes in many plant species (Friedrich et al. 1996). Activation of genes related to SAR was also demonstrated in ASM-treated apple seedlings 2 to 7 dat; levels of PR-1 and PR-8 mRNA were increased 10-fold and PR-2 mRNA 100-fold in ASM-treated compared to untreated seedlings (Maxson-Stein et al. 2002). Thus, ASM induces the same set of SAR genes that is activated by SA, and it induces the same spectrum of disease resistance that is mediated by the SA-dependent pathway (Ryals et al. 1996). The present

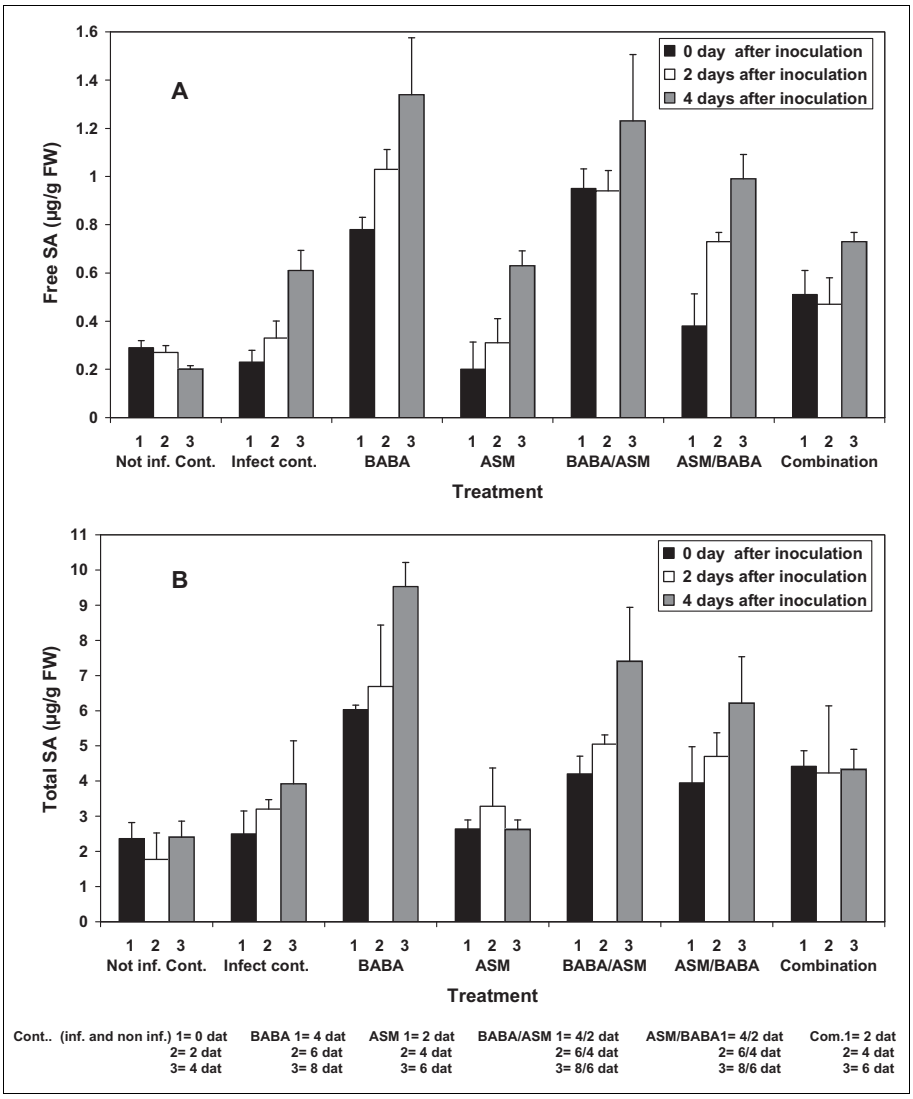


Fig. 5: Accumulation of free (Fig. 5A) and total (Fig. 5B) salicylic acid (SA) ((µg g^{-1} FW) in inoculated leaves of apple seedlings. Apple seedlings were treated with BABA (1.0 mg ml^{-1}) and ASM (0.1 mg ml^{-1}) alone, in sequence as well as in combination. Plants were inoculated with *Erwinia amylovora* 4 dat with BABA or 2 dat with ASM. Means of standard deviation for five seedlings per treatment are shown.

results showed that foliar treatments with ASM or BABA alone or combined treatment with BABA and ASM (BABA/ASM) induced significant increases of peroxidase (PO) activities at all sampling times, the highest levels of PO were determined 2 and 4 dat with ASM. PO levels increased more slowly after BABA treatment and reached 6 dat the highest activity. Several studies reported an accumulation of PO in ASM-treated apple and pear leaves which were protected against fire blight and scab diseases (Baysal et al. 2002; Faize et al. 2004). Chamsai et al. (2004) and Cohen (2002) found accumulation of phenolic compounds in BABA-treated tomato plants. Thus, it may also be suggested that BABA treatment may enhance the level of phenolic compounds in leaves of BABA-treated apple seedlings. Accumulation of phenolic compounds in infected apple tissues has been reported by Roemmelt et al. (1999). High contents of phenolic acids in apple leaves might act as a chemical barrier inhibiting the spread of the bacterial pathogen throughout the cortex tissue of petiole and stem. It is suggested that phenolic compounds may play an essential role in restricting spreading of the bacterial pathogen *E. amylovora*.

The results indicate that the effectiveness of BABA or ASM against fire blight was improved when the compounds were applied in sequence. These findings suggest additive or synergistic relationship in activity of both compounds. Similar results have been reported in several other studies. It was reported that BABA acts synergistically with ASM or chitosan (Cohen 2002). Our data show that combined treatments of BABA and ASM were more effective in inducing resistance against fire blight when BABA was applied before ASM (BABA/ASM). This effect may be associated with an enhanced capacity to activate induced cellular defense responses – a process called 'priming' (Conrath et al. 2002).

The data indicate that the resistance inducers not only suppressed symptoms but also retarded bacterial multiplication *in planta*. It may be assumed that reduction of bacterial growth in BABA/ASM-treated seedlings was accompanied by accumulation of defense constituents in plant tissue, such as acidic PR proteins, phenolic acids, peroxidases, lignin and other defense constituents. Reduced growth of bacterial pathogens was also reported from *Arabidopsis* plants induced with ASM and challenged with *Pseudomonas syringae* pv. *tomato* (Lawton et al. 1996). In conclusion, it is suggested that BABA/ASM applied at certain intervals and streptomycin treatment at critical times during bloom should be an effective strategy for managing blossom and shoot infections.

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