Baysal, Ö.[1*], Gölükcü, Ş.B.[1], Ünlü, A.[1], Zeller, W.[2]
[1]West Mediterranean Agricultural Research Institute (BATEM) Plant Pathology, Department P.B. 35, 07100 Antalya, Turkey, [*]Correspondent author: E-Mail: obaysal@ttnet.net.tr
[2]Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für biologischen Pflanzenschutz, Heinrichstr. 243, 64287 Darmstadt, Germany

An early oxidative burst in apple rootstocks treated with DL-β-amino butyric acid (BABA) against fire blight (*Erwinia amylovora*)

Abstract

Systemic acquired resistance (SAR) was induced by pre-treatment with the chemical inducer DL-β-amino butyric acid against fire blight disease caused by *Erwinia amylovora* (Ea 7/74). The plants were inoculated with 10^8 cfu/ml bacterial suspension, and disease development was evaluated up to 14 days post inoculation. Although *in vitro* growth of bacteria was not affected by DL-β-amino butyric acid treatment, its pre-inoculation application (500 µg/ml) significantly reduced disease severity and bacterial population. DL-β-amino butyric acid treated plants showed significantly higher H_2O_2 generation, compared to untreated plants. The findings indicate that pre-treatment with the chemical inducer DL-β-amino butyric acid activated H_2O_2 generation *in planta* more strongly when the plants were challenged with the pathogen; this may be associated with induction of plant resistance to bacterial pathogens and an effect of BABA in modulation of pathogen defence pathways.

Introduction

Fire blight is one of the most serious diseases affecting apple and pear trees as well as other *Rosaceae* plants (van der Zwet and Beer 1992). Fire blight attacks all aboveground organs of the host plants often leading to their death. Chemical control of the disease relies upon the use of antibiotics (such as streptomycin) and copper compounds which prevent bacterial multiplication and further infection. Unfortunately, the antibiotics lead to the selection of resistant bacterial populations and therefore their use is strictly limited or even forbidden in several countries. In addition, none of these chemicals is systemic, and to be effective, they have to be applied on the whole plant surface before the pathogen enters the plant tissues. The induction of plant resistance may be one of the potential methods of reducing the severity of disease caused by the pathogen.

Pre-treatment of susceptible plants with avirulent pathogens (biotic inducer) can enhance resistance to subsequent attack not only at the site of treatment, but also in tissues distant from the initial infection sites. Typically, this inducible resistance system known as systemic acquired resistance (SAR) is effective against diverse pathogens including viruses, bacteria and fungi (Ryals et al. 1996). SAR is characterized by a reduction in the number and severity of lesions following challenge inoculation with a normally virulent pathogen. In addition to biotic inducers, certain chemicals with no direct anti-microbial effect can also induce SAR in plants. Natural products such as salicylic acid (SA) and synthetic chemical compounds, such as 2,6-dichloroisonicotinic acid (INA), may serve as good alternatives to classical pesticides depending on their efficacy. Both SA and INA, however, are not tolerated by some plants (Ryals et al. 1999). Potassium salts, acibenzolar-S-methyl, and amino butyric acid (BABA) were reported to induce SAR in plants (Cohen et al. 1999; Narusaka et al. 1999; Oostendorp et al. 2001; Baysal et al. 2003; Baysal and Zeller 2004; Baysal et al. 2005). In our previous study (Baysal et al. 2005) BABA and the effect of its different doses were tested against bacterial canker disease in tomato and the suppressive effect was found to be 52%. The aim of the present study was to test BABA for its ability to induce resistance in apple rootstocks against Ea.

Material and methods

Plant material: Young, greenhouse-grown apple rootstocks (M9 Bursa, Turkey) were used for all experiments. These rootstocks are highly susceptible to fire blight caused by *E. amylovora*. Plants were grown in 10 cm pots in a soil mix containing sand, perlite, and peat compost under greenhouse conditions at 25 ± 5 °C with 68-80% RH. The soil mix also contained a slow-release fertilizer (14-12-14 N-P-K). Plants were watered daily. 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.

Bacterial strain and inoculation: The bacterial strain of *E. amylovora* (Ea7/74) was obtained from the Federal Biological Research Centre, Germany (BBA). Inoculum suspension was prepared from early log-phase cells, which were obtained by growing the bacterial strain in nutrient 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). Their concentration was adjusted to10^8 cfu/ml by dilution to give an OD_{660} of 0.1. The two youngest leaves of the seedlings were cut at the tip and inoculated by dipping into a suspension of 10^8 cfu/ml, as described by Baysal and Zeller (2004).

Application of BABA: BABA was dissolved in distilled water to obtain a concentration of 500 µg/ml BABA, and was sprayed (ca. 200 µl per seedling) on whole rootstocks. After the treatment, the rootstocks were maintained in a greenhouse as described above. Application on M9 rootstocks before inoculation control plants were sprayed with water at the same intervals. Plants of the first group were treated with BABA alone, the second group with BABA and inoculated with a bacterial suspension 3 days after treatment. The plants of the control group were treated with water as described. The level of the resistance induced in apple seedlings against *Ea* was evaluated at 4, 7, 10 and 14 days after inoculation (dai) by using a 0-10 arbitrary scale (Baysal and Zeller 2004). A mean disease severity index (DSI) was calculated from each treatment by summing the score of the 60 plants (three replicates of 20 plants for each treatment), and expressing the value as percentage according to Anfoka (2000). Mean percentage protection for each treatment was calculated as previously described (Godard et al. 1999): [(DIw x DIt) /DIw)] x 100 where DIt is the mean DI of the treatment and DIw the mean DI of the water control.

Effect of BABA on bacterial growth in planta: Bacterial colony forming units (cfu) were recovered from inoculated tissues, treated with either BABA or water 3 days before inoculation, by removing inoculated shoot tips (1g plant material) which were homogenized in 0.06% NaCl solution (1:1), diluted serially from 10^{-1} to 10^{-6} and plated on the modified Miller-Schroth medium (Brulez and Zeller 1981). Aliquots of alternate dilutions were plated on NYA agar plates. Plates were incubated at 27 ^oC for 48 h, and emerging colonies counted on all dilution plates showing bacterial growth. Bacterial numbers *in planta* were calculated for each of the dilution plates, and a mean value was obtained from replicates. Each dilution from each leaf disc was duplicated. Results presented are means of two separate experiments in which three leaf discs from each treatment were homogenized.

Assay of hydrogen peroxide concentration: The concentration of H_2O_2 in the leaves was determined according to a modified method of Capaldi and Taylor (1983). Leaves were ground in 5% TCA (2.5 ml per 0.5 g leaves tissues) with 50 mg active charcoal at 0°C and centrifuged for 10 min at 15000g. The supernatant was collected, adjusted with 4 N KOH to pH 3.6 and used for the H_2O_2 assay. The reaction mixture contained 200 µl of leaf extract, 100 µl of 3.4 mM 3-methylbenzothiazoline hydrazone. The reaction was initiated by adding 500 µl of horseradish peroxidase solution (90 U 100 ml-1) in 0.2 M sodium acetate (pH 3.6). Two minutes later 1400 µl of 1 N HCl was added. The Extinction at 630 nm was read after 15 min.

Experimental design and statistical analyses: The experiment was arranged in a completely randomized split-plot design with three replicates of 20 plants per treatment. Data obtained on various numbers of days after inoculation, and which usually included typical disease development, are presented. All experiments were repeated at least twice. Standard analyses of variance (ANOVA) were carried out by using the SPSS Statistical computer software program (Version 10). ANOVA was performed to analyze

the data, and the significance of differences among treatments was determined according to Duncan's Multiple Range Test ($P<0.05$).

Results

The effect of BABA on disease resistance: Resistance induced in apple rootstocks by BABA is shown in Fig. 1. Initial symptoms appeared on control plants as small marginal wilting 4 days after inoculation. The mean disease severity index (DSI) in these plants was 11%. The progress of the disease in control plants increased with time and by 14 dai most of the plant leaves developed severe symptoms. The mean DSI in these plants reached 80%. After application of BABA, a remarkable reduction in the disease severity index occurred (Figure 1). The time between initial treatment with BABA and subsequent inoculation with Ea7/74 significantly affected the efficacy of the induced resistance. Although all interval times significantly reduced the disease index, the greatest disease suppression was caused by BABA treatment 3 days before inoculation (Figure 1). The resistance induced by the BABA treatment was already evident 4 dai and lasted for the entire experimental period (until 14 dai). Untreated plants showed a significantly faster disease development during this period. The disease index was reduced in BABA-treated rootstocks 7 dai, and this was maintained at the same level until 14 dai. Disease indices of control seedlings were 80% whereas those of BABA-treated seedlings were only 53% at 14 dai. Since the highest induced resistance was observed at a time interval of 3 days between treatment and inoculation, this interval was taken into consideration in order to determine the bacterial growth and analysis of H_2O_2 generation.

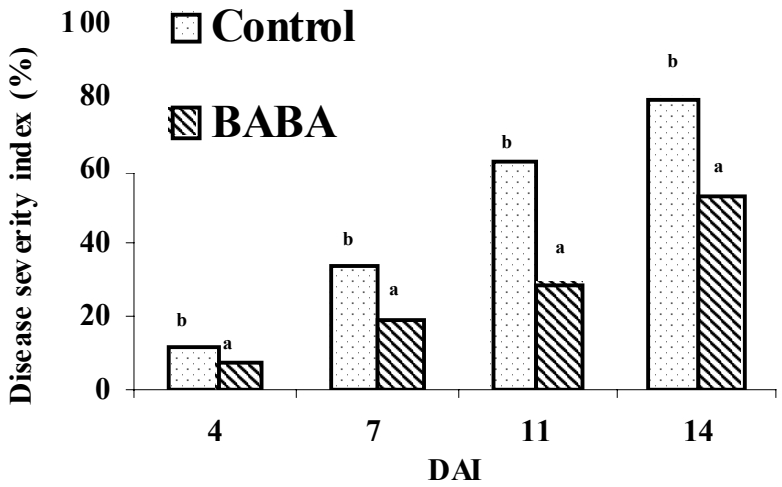

Fig. 1 Effect of BABA on the severity of the disease symptoms caused by *E. amylovora* 7/74. After treatment with different plant activators or water (control), rootstocks were inoculated 72 h later with the Ea 7/74. Inoculated leaves were scored at 4, 7, 11 and 14 days after inoculation (dai) using the 0-10 scale as described in Material and methods section. A mean disease severity index (DSI%) was calculated from each treatment by summing the score of the 30 plants (two replicates of 15 plants per treatment), and expressing the value as a percentage according to Anfoka (2000). Data are presented as the mean of the two independent experiments. Bars with the same patterns which have the different letters are significantly different according to Duncan's Multiple Range Test ($P<0.05$).

Bacterial multiplication *in planta:* The growth of Ea was markedly reduced in BABA-treated rootstocks, compared to the water-treated control (Figure 2). This inhibitory effect was first observed 4 dai and monitored until 11 dai. The bacterial population was reduced by BABA, compared to control plants at 4, 7 and 11 dai, respectively (Figure 2).

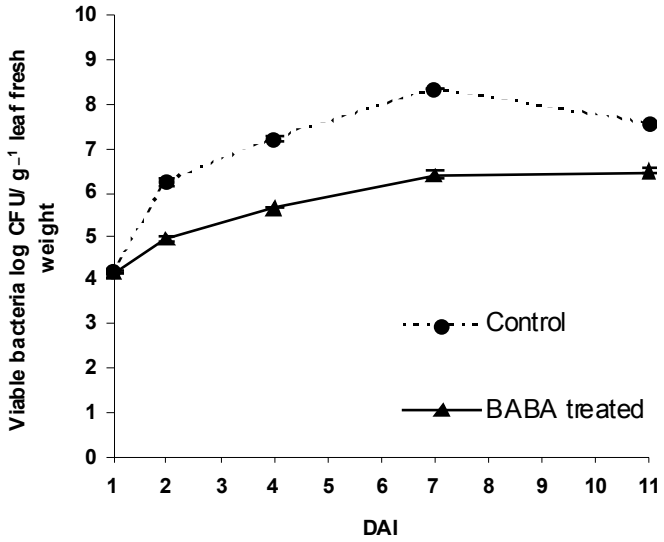

Fig. 2 Effect of BABA on bacterial growth of *E. amylovora* in apple rootstocks. Three days after treatment with BABA or water, rootstocks were inoculated with Ea7/74. Data are means values for three leaf discs, and the bars represent standard deviations. Inhibitory effect of BABA treatment on bacterial growth was significantly different after inoculation according to Student's two-sample t-test ($P < 0.05$).

Assay of hydrogen peroxide concentration: The data showed that BABA leads to increasing H_2O_2 generation in plants when the plants were challenged with pathogens. Although the plants, when sprayed with BABA alone, showed no significant increase in H_2O_2 generation up to 24 h post inoculation, BABA-treated and inoculated plants showed significantly higher H_2O_2 generation up to 48 h post inoculation (Figure 3).

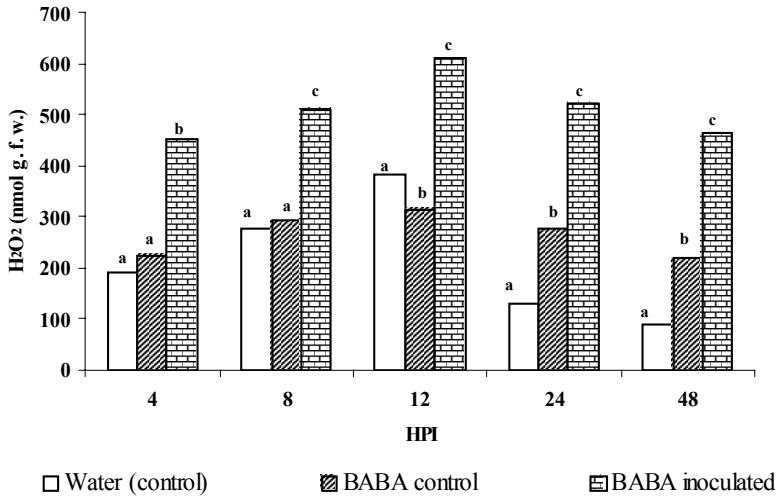

Fig. 3 H_2O_2 concentration (nmol g^{-1} FW) in BABA-treated and water-treated apple rootstocks inoculated with Ea over 48 h. Leaves were treated with BABA at 72 h before inoculation. For controls, leaves were sprayed with water at 72 h before inoculation. The values (± standard deviations) of 4 different samples with the same letters represent values that are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$)

Discussion

The current study assessed the effect of the plant activator BABA on disease development caused by Ea. Results confirm that BABA induces resistance in apple rootstocks. Apart from this study, amino butyric acid has been described in tomato against fungal pathogens (Cohen et al. 1994). For the development of

resistance plants need an interval period before being challenged with a pathogen. In most cases this interval was reported to be between 1 and 7 days. In plants, the increased production of both the superoxide radical and H_2O_2 is a common feature of defence responses to challenge by avirulent pathogens and elicitors (Lamb and Dixon 1997). It may be hypothesised that a low nutrient concentration or/and accumulation of antimicrobial compounds in the intercellular space of treated leaves of apple rootstock tissues, where bacteria grow, or cell wall alterations such as physiological barriers in the xylem tissues may be a limiting factor for bacterial growth. BABA dependent resistance does not appear to be due to an antimicrobial effect of the compound, BABA seems to be a useful tool for induced resistance studies in apple as observed in other plant species. Biocontrol organisms, improved varieties and BABA will provide the farmer with a new option for disease control. We believe that the future use of BABA will have a profound impact combined with lower doses of conventional copper compounds, on the control of fire blight disease.

References

Anfoka, G.H. 2000. 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 Protection 19: 401-405.

Baysal, Ö., Zeller, W. 2004. Extract of *Hedera helix* induces resistance on apple rootstock M26 similar to acibenzolar-S-methyl against fire blight (*Erwinia amylovora*). Physiol. Mol. Plant Pathol. 65: 305-315.

Baysal, Ö., Soylu, S., Soylu, E.M. 2003. Induction of defence-related enzymes and resistance by the plant activator acibenzolar-S-methyl in tomato seedlings against bacterial canker caused by *Clavibacter michiganensis* ssp. *michiganensis*. Plant Pathol. 52: 747-753.

Baysal, Ö., Gürsoy, Y.Z., Örnek, H., Duru, A. 2005. Induction of oxidants in tomato leaves treated with DL-β-amino butyric acid (BABA) and infected with *Clavibacter michiganensis* ssp. *michiganensis* Eur. J. Plant Pathol. 112: 361–369.

Brulez, W., Zeller, W. 1981. Seasonal changes of epiphytic *Erwinia amylovora* on ornamentals in relation to weather conditions and course of infections. Acta Hortic. 117: 37-43.

Capaldi, D.J., Taylor, K.E. 1983. A new peroxidase color reaction: oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) with its formaldehyde azine. Application to glucose and choline oxidases. Analyt. Biochem. 129: 329-336.

Cohen, Y., Niederman, T., Mösinger, E., Fluhr, R., 1994. β-aminobutyric acid induces the accumulation of pathogenesis-related proteins in tomato (*Lycopersicon esculentum* L.) plants and resistance to late blight infection caused by *Phytophthora infestans*. Plant Physiol. 104: 59-66.

Cohen, Y., Reuveni, M., Baider, A. 1999. Local and systemic activity of BABA (DL-3-aminobutyric acid) against *Plasmopara viticola* in grapevines. Eur. J. Plant Pathol. 105: 351-361.

Godard, J.P., Ziadi, S., Monot, C., Le Corre, D., Silue D. 1999. Benzothiadiazole (BTH) induces resistance in cauliflower (*Brassica oleracea* cv. *botrytis*) to downy mildew of crucifers caused by *Peronospora parasitica*. Crop Protection 18: 397-405.

Lamb, C.J., Dixon, R.A., 1997. The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 251-275.

Mehdy, M.C., Sharma, Y.K., Sathasivan, K., Bays, N.W. 1996. The role of activated oxygen species in plant disease resistance. Physiol. Plant. 98: 365–374.

Narusaka, Y., Narusaka, M., Horio, T., Ishii, H. 1999. Comparison of local and systemic induction of acquired disease resistance in cucumber plants treated with benzothiadiazoles or salicylic acid. Plant Cell Physiol. 40: 388-395.

Oostendorp, M., Kunz, W., Dietrich, B., Staub, T. 2001. Induced disease resistance in plants by chemicals. Eur. J. Plant Pathol. 107: 19–28.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., Hunt, M.D. 1996. Systemic acquired resistance. Plant Cell 8: 1809-1819.

Van der Zwet, T., Beer, S.V. 1992. Fire blight - its nature, prevention and control: a practical guide to integrated disease management. U.S. Dept. Agric. Inf. Bull. 631.