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ORIGINAL RESEARCH

Activation of tomato plant defence responses against bacterial wilt caused by *Ralstonia solanacearum* using DL-3-aminobutyric acid (BABA)

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Abstract In this study, we investigated the ability of DL-3-aminobutyric acid (BABA) to protect tomato against bacterial wilt caused by *Ralstonia solanacearum*. This was combined with studies of accumulation of total phenolic compounds, free and total salicylic acid (SA), and activity of enzymes related to plant defence, i.e., polyphenol oxidase (PPO) and catalase (CAT). Under greenhouse conditions, tomato plants pre-treated by soil drenching with BABA profoundly reduced disease severity of bacterial wilt compared to plants receiving a soil drench with water. Thus, BABA reduced leaf wilting index by 75.3 % and vascular browning index by 69.9 %, without any *in vitro* inhibitory activity on the pathogen. BABA treatment significantly reduced the population of *R. solanacearum* in stems of tomato plants and additionally also significantly increased both fresh and dry weight of roots and shoots of tomato plants compared with the inoculated control. Application of BABA resulted in a high increase in PPO activity both in plants with and without inoculation. Compared to water-treated plants, treatment with BABA also induced a significant increase of total phenolic compounds as well as of free and total SA in leaves of both inoculated and non-inoculated tomato plants at all sampling times. CAT activity decreased in tomato plants treated with BABA in comparison with the water-treated control plants and the decrease in activity correlated with an increasing total SA accumulation. These findings suggest that BABA treatment resulted in induction of resistance to bacterial wilt in tomato.

Keywords Catalase (CAT) · Phenolic compounds · Polyphenol oxidase (PPO) · Salicylic acid (SA)

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Introduction

Tomato (*Lycopersicon esculentum* Mill) is one of the most common and important vegetable crops in the world. Yield losses of tomatoes caused by soil and seed infection by soil-borne pathogens give problems wherever tomato is grown. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici*, damping-off caused by *Pythium* spp. or *Rhizoctonia solani* as well as bacterial wilt caused by *Ralstonia solanacearum* are limiting factors for production of tomatoes (Amini 2009; Chen et al. 2009). Bacterial wilt caused by the soil-borne bacterium *R. solanacearum* is a serious disease in the tropics and in warm climate regions in the world, causing severe losses in many agricultural crops (Hayward 1991). Many studies have been carried out to control the disease, but without great success. No promising control of bacterial wilt has been achieved using antibiotics, soil fumigants or resistance breeding. Other control methods such as soil amendments,

crop rotations, biological control, and field sanitation are often not effective (Ji et al. 2005). In addition, the use of copper compounds, which are widely used for control of plant bacterial diseases, will be limited in many countries, especially in the European Union countries. Therefore, it is necessary to find more selective and safe materials for management of bacterial wilt on tomato.

One of the methods of reducing bacterial diseases is the induction of plant resistance. Induced resistance (IR) as a general phenomenon in plants has been studied in many host plant-pathogen-interactions. Plants can be induced to enhance their defence against pathogen infection by treatment with various biotic and abiotic inducers (Walters et al. 2005). The use of chemicals as inducers of resistance aims at developing new compounds for disease control by meeting the requirements for safe application under greenhouse and fields conditions, i.e., easy application; no direct toxicity to pathogens; no toxicity to plants; no negative effects on plant growth; broad spectrum activity against pathogens and low economical cost for farmers (Edreva 2004). Thus, the application of chemical inducers of resistance is an exciting method to supplement the classical chemical means of disease control by providing both effective and ecologically-friendly plant protection.

Abiotic compounds such as DL-3-aminobutyric acid (BABA) have been reported to induce resistance in a variety of plants against a wide range of microbial pathogens without possessing any direct antimicrobial activity. It is a simple, non-protein amino acid which, when sprayed onto the leaf surface or drenched into the soil, induces resistance against various foliar and root pathogens (Jakab et al. 2001; Cohen 2002). BABA was shown to induce resistance in tomato plants against fungal pathogens like *Botrytis cinerea* (Cohen 2002), *Fusarium oxysporum* f. sp. *lycopersici* (Chamsai et al. 2004) and the nematode *Meloidogyne javanica* (Oka et al. 1999). In case of bacterial pathogens in tomato plants, some reports indicate that pre-treatment with BABA is effective against *Xanthomonas vesicatoria* (Cohen 2002) and *Clavibacter michiganensis* subsp. *michiganensis* (Baysal et al. 2005). BABA induces resistance against diseases through either the activation of the salicylic acid (SA) signalling pathway or through the activation of the jasmonic acid or ethylene signalling cascade (Zimmerli et al. 2001).

To the best of the authors' knowledge, there is little information concerning the use of BABA against bacterial wilt of tomato caused by *Ralstonia solanacearum* (RS). Therefore, we studied its effect against bacterial wilt under greenhouse conditions. We also followed the changes in polyphenol oxidase (PPO), catalase (CAT), total phenolic compounds and free and total salicylic acid (SA) as markers of induced resistance by relating their levels and temporal occurrence to the application of BABA and reductions in infection by the pathogen.

Materials and methods

Tomato plants

Seedlings of tomato plants cv. Super Jackal were cultivated in a greenhouse in plastic pots (diameter 25 cm) filled with Humosoil®- (European Compost Network ECN e.V. Bochum, Germany) sand mixture (3:1, v/v). The plants were grown at 25 °C during the day (14 h/day) with a 14 h photoperiod (Philips, Son-Tagro 400 W) and at 20 °C during night (10 h/day). Plants were fertilized weekly with 0.3 % Wuxal-Super®-liquid fertilizer (Aglukon Spezialdunger GMBH, Düsseldorf, Germany) [N:P:K, 8:8:6+microelements (Gabi plus)]. Four week-old tomato seedlings with four-six fully expanded leaves were used in all experiments.

Bacterial pathogen, inoculum preparation and method of inoculation

Ralstonia solanacearum, isolate RS7, isolated from naturally infected tomato plants with bacterial wilt symptoms, was collected from Assiut Governorate, Egypt, and used in these studies. A stored stock culture of this isolate was streaked on TTC medium in Petri dishes and incubated at 27 °C for 48 h (Kelman 1954). One g of 2,3,5 triphenyl tetrazolium chloride was dissolved in 100 ml distilled water as a TTC stock solution. After autoclaving, casamino acid-peptone glucose agar (CPG) medium was cooled to 55 °C and 5 ml of a 1 % stock solution of TTC was added. The CPG agar medium consisted of 5.0 g glucose, 10.0 g peptone, 1.0 mg casamino acid, 20 g agar and 1000 ml distilled water, with pH adjusted to 7.0. A single colony of the isolate was selected and grown in 250 ml Erlenmeyer flasks containing 100 ml of nutrient sucrose broth (5.0 g peptone, 3.0 g beef extract, 5.0 g sucrose and 1000 ml distilled water, with pH adjusted to 7.0) and incubated at 27±2 °C for 48 h on a rotary shaker (ZHWY-211C, Selecta, Spain) at 150 rpm. Bacterial suspension cultures were centrifuged for 8 min.

at 8000 g, the cells re-suspended in sterile distilled water and cell density adjusted to be 10^8 cfu/ml using a spectrophotometer (Spectronic® 20 GenesysTM, Schutt Labortechnik) at λ=620 nm.

For tomato root inoculations, an alcohol-flamed knife was inserted 5–10 cm deep into the soil of each pot to cut the roots along two sides and inoculation was performed by soil drenching with 30 ml of the bacterial suspension, which was added to each pot around the basis of each plant. Control plants were treated with the same volume of distilled water. Prior to inoculation, plants were not watered for 24 h. The inoculated and non-inoculated tomato plants were kept in the same greenhouse as before and watered regularly with tap water. During the experimental period, tomato plants were fertilized weekly with 0.3 % Wuxal-Super®-liquid fertilizer (N:P:K, 8:8:6+microelements).

Inhibition assay

Using the impregnated filter paper disk method (Sholberg et al. 2001), the toxicity of 1.0 mg/ml BABA (Fluka Chemie GmbH,Buchs, GA13766, Switzerland) against *R. solanacearum* (isolate RS7) was tested. One ml bacterial suspension of RS7 (3×10^9 cfu/ml) from 24 h old cultures was added to 50 ml of sterilized TTC medium in Erlenmeyer flasks (100 ml) at 47 °C and mixed. The mixture was then poured in four Petri dishes (9 ml in diameter). After solidification of the medium, sterilized Whatmann standard filter paper disks (9 mm diameter, 1 mm thick; Schleicher and Schuell GmbH), to which 50 μl of BABA had been pipetted, were placed in the middle of the seeded agar surface. Streptomycin sulphate (Sigma) (0.5 mg/ml) and sterile water were used as positive and negative controls, respectively. Four replications were used for each treatment and they were repeated twice. To prolong the diffusion time of the compounds in the agar medium, the plates were first incubated at 4 °C for 24 h and then at 28 °C for 48 h. After incubation, inhibition zones around each disk were measured and the area of inhibition expressed in mm^2.

BABA application, disease assessment and determination of bacterial population

Tomato plants were treated by soil drenching with 50 ml per pot of BABA (0.5 mg/ml).

Pots were divided into four groups and treated as follows:

1. Treatment with BABA two days before inoculation with *R. solanacearum* to serve as induced, inoculated treatment (+BABA+RS).
2. Treatment with BABA to serve as inducer control, without inoculation (+BABA -RS).
3. Treatment with tap water and inoculated with *R. solanacearum* to serve as the inoculated control (-BABA+RS).
4. Treatment with tap water to serve as non-inoculated control (-BABA-RS).

Three weeks after inoculation, the severity of wilt symptoms on leaves and vascular browning symptoms of RS-infection were scored as leaf wilting index (LWI) and vascular browning index (VBI).

Wilt symptoms on leaves were classified using the following 0–5 rating scale: 0: no symptoms; 1: up to 10 % of the leaf area wilted; 2: 11–40 % of the leaf area wilted; 3: 41–60 % of the leaf area wilted, 4: 61–85 of the leaf area wilted and 5: > 85 % of the leaf area wilted or leaf is dead.

LWI was calculated as follows:

$$\text{LWI}\ (\%) = \frac{\text{Sum of ratings}\ (0-5\ \text{scale})}{5 \times \text{number of leaves evaluated}} \times 100$$

The vascular browning symptoms of tomato stems were classified using a 0–3 rating scale. Each internode of the stem was cut transversely and disease severity assessed. 0: no browning of xylem vessels in internodes; 1: one xylem vessel in the internode brown; 2: two xylem vessels brown and 3: all three xylem vessels brown.

VBI was calculated as follows:

$$\text{VBI}\ (\%) = \frac{\text{Sum of ratings}\ (0-3\ \text{scale})}{3 \times \text{number of internodes evaluated}} \times 100$$

Plants were then used to assess the bacterial multiplication in their tissues. One g of the lower stem internodes (15 to 20 cm above the soil) from each treatment was washed with tap water, surface sterilized with 3 % sodium hypochlorite and washed with sterile water. Stem tissues were homogenized in a sterile mortar and pestle with 10 ml 0.1 M potassium phosphate buffer (pH 7.0). Stem homogenates were serially diluted from 10^{-1} to 10^{-9} with 0.1 M potassium phosphate buffer. A volume of 200 μl of each

dilution were transferred to a Petri dish with TTC medium and spread by using a glass rod. Plates were incubated at 27 °C for 48 h in darkness and the number of bacterial colonies counted.

Effect of BABA on disease severity and tomato growth under greenhouse conditions

Two days after treatment with 50 ml BABA (0.5 mg/ml) or water, tomato plants were inoculated with *R. solanacearum* RS7 as described before. After 3 weeks, leaf wilting index (LWI) and vascular browning index (VBI) were calculated. In addition, the fresh and dry weights of shoots and roots were determined. Shoots were cut at soil level, weighed and placed in paper bags. Roots were carefully washed in running water and blotted dry with paper towels and weighed. Shoots and roots were dried in an oven for 4 days at 70 °C. For each treatment, four plants were used as replications (one plant per pot). The experiment was repeated three times.

Preparation of samples for physiological assays

Tomato plants were treated by soil drenching with 50 ml/pot of BABA (0.5 mg/ml). Two days after treatment, one half of the treated tomato plants was inoculated with *R. solanacearum* as described before (+BABA+RS) and the other half of the treated plants was not inoculated (+BABA-RS). Inoculated (-BABA+RS) and non-inoculated control (-BABA-RS) plants were treated with 50 ml/pot of water. Immediately before treatment (0 days after treatment) as well as 2, 4, 6 and 8 days after treatment with BABA, leaves of tomato plants were sampled separately for determination of total phenolic compounds, free and total salicylic acid (SA), catalase (CAT) and polyphenol oxidase (PPO) activities.

Extraction of enzyme and protein analysis

For measuring PPO and CAT activities, 0.5 g leaf tissue from tomato plants was frozen in liquid nitrogen, ground in a mortar and homogenized in 5 ml 10 mM potassium phosphate buffer (pH 7.0) containing 4 % (w/v) polyvinylpyrrolidone (Sigma). The homogenate was centrifuged at 12,000×g for 30 min and the supernatant obtained was used for the assays. All steps in the preparation of the extract were carried out at 0–4 °C. The supernatants (crude enzyme extract) were stored at −60 °C or immediately used for determination enzymes activities and total protein. For all enzymes under investigation, each treatment consisted in four replications (plants) and two spectrophotometric readings were taken per replication using a spectrophotometer (Spectronic® 20 GenesysTM, Schutt Labortechnik). An aliquot of the extract was used to determine the protein content using the method of Bradford (1976) using bovine serum albumin (Sigma Diagnostics St. Louis, Mo, USA) as standard.

Catalase (CAT) assay

The CAT activity was assayed by measuring the rate of disappearance of H_2O_2 at 240 nm using the method of Kato and Shimizu (1987). Three ml reaction mixture containing 10 mM potassium phosphate buffer (pH 7.0), 0.1 ml crude extract and 0.035 ml 20 mM H_2O_2 were used. The decrease in H_2O_2 was followed as the decline in absorbance at 240 nm, and the specific activity was calculated using the extinction coefficient [40 mM^{-1} cm^{-1} at 240 nm] for H_2O_2 (Kato and Shimizu 1987).

Polyphenol oxidase (PPO) assay

In assaying for PPO activity, 1 ml reaction mixture contained 0.5 ml crude extract (20 μg protein), 25 mM citrate-phosphate (pH 6.4) and 5 mML-proline (Sigma). Each sample was aerated for 2 min in a small test tube followed by the addition of pyrocatechol (1,2-dihydroxybenzene, Sigma) as the substrate at a final concentration of 20 mM. The increase in absorbance at 520 nm was recorded for 1 min at 25 °C using the same spectrophotometer as before. PPO activity was expressed as the change in absorbance of the reaction mixture per mg of total protein per min (Mohammadi and Kazemi 2002).

Extraction of total phenolic compounds and salicylic acid

For determining the concentration of total phenolic compounds and free and total SA, leaf extracts were prepared according to the method of Malamy and Klessig (1992) with some modifications. One g of tomato leaves was frozen in liquid nitrogen, ground in a mortar with 0.9 g sea sand and homogenized in 3 ml 90 % methanol (Labscan). The mortar was washed twice with 2 ml 90 % methanol. The combined homogenates were centrifuged for 15 min at 4 °C at 12,000 g and the supernatants collected. The pellet was re-

extracted with 4 ml 90 % methanol. After centrifugation, the supernatants were combined and the solvent evaporated at 40 °C under vacuum. The residues were re-suspended in a mixture of 100 μl 5 % trichloroacetic acid (Sigma) and 1 ml 99.8 % methanol (w/v). The volume of each extract was adjusted with distilled water to 5 ml and centrifuged at 8000×g for 10 min. The supernatants of the extracts were used for measuring both total phenolic compounds and free and total SA.

Determination of total phenolic compounds

One ml of the methanol supernatant, prepared as described above, was added to 5 ml distilled water and 250 μl Folin-Ciocalteu reagent (Merck), and the solution was incubated at room temperature. After 3 min, 1 ml of a saturated solution of Na_2CO_3 and 1 ml distilled water was added and the reaction mixture was incubated for 1 h. The absorbance of the developed blue colour was measured at 725 nm using a blank of water and reagent only. Caffeic acid (Fluka) was used as reference phenolic compound. The total phenolic compounds in the samples were expressed as milligram caffeic acid per g of leaf fresh weight (Malick and Singh 1980).

Quantification of free and total salicylic acid

Free SA content was determined in the supernatants of the extracts, prepared as described above, using a high-performance liquid chromatography (HPLC) system equipped with a ProStar 230 quaternary pump and a model 410 autosampler (Varian, Walnut Creek, CA, USA) coupled to a fluorescence detector (Jasco FP1520, Jasco International, Tokyo, Japan). Fluorescence was measured at λ=304 and 408 nm for excitation and emission, respectively. The concentrations of SA in samples were calculated and evaluated in an external standard mode using a Varian Galaxy data system (Agilent Technologies, Santa Barbara, California). Total SA was determined using samples of the extracts, prepared as described above. One ml of the extracts was hydrolysed for 2 h at 80 °C after addition of 250 μl 32 % HCl to release glucosidically bound SA. After acid hydrolysis, the pH of the extracts was adjusted to 6.0 with a solution of 2 M NaOH and 1 M $NaHCO_3$. The total SA contents were determined by HPLC as mentioned above. The recovery rates were estimated by extracting tomato leaf tissue to which a known amount of SA had been added.

Experimental design and statistical analysis

The greenhouse experiments and sampling of plants were performed twice and data collected were analysed separately for each time point. The data obtained in the individual experiments showed the same statistical trend when analysed. Moreover, correlation coefficients were calculated between data from the two experiments for each parameter and the correlation coefficients were>0.90. Consequently, only the data of the first experiment are presented in the manuscript. The experiments to determine leaf wilting index (LWI), vascular browning index (VBI), fresh weight (FW) and dry weight (DW) were arranged in a completely randomized design with four replications of plants (one plant/pot) per treatment. For the enzyme and physiological investigations, experiments in the greenhouse were designed as a spilt-plot design with four replications (plants). Treatments (+BABA+RS, -BABA+RS, +BABA-RS, -BABA-RS) were arranged in the main plot and the sampling time for enzyme activity or physiological determination (0, 2, 4, 6, and 8 days after treatment) in the sub-plots. For each replication, two spectrophotometric readings were taken for each enzyme and total phenolic compounds and two readings of HPLC for SA. Data were analysed by the statistical analysis system MSTAT-C (Version 2.1). Means were compared with a Least Significant Difference (LSD) test at $P \leq 0.05$.

Results

Effect of BABA on leaf wilting index (LWI), vascular browning index (VBI) and bacterial population (BP)

Soil drenching with BABA (+BABA+RS) significantly reduced disease severity of bacterial wilt on tomato plants compared to the untreated inoculated control (-BABA+RS). Although the tested dose of BABA (0.5 mg/ml) showed no toxic effects on growth of *R. solanacearum in vitro* and no phytotoxic effects on tomato plants *in vivo* (data not shown), BABA treatment resulted in a significant reduction of bacterial wilt (Table 1). Pre-treatment of tomato seedlings with BABA reduced both LWI and VBI by 75.3 and 69.9 %, respectively (Table 1).

Three weeks after inoculation, the bacterial population was significantly higher in stem sections of -

BABA+RS plants compared to+BABA+RS plants (Table 1).

Effect of BABA on fresh weight (FW) and dry weight (DW)

FW and DW of shoots and roots of tomato plants inoculated with *R. solanacearum* were significantly lower than those of non-inoculated control plants (Fig. 1). Comparison of FW and DW of shoots and roots of -BABA+RS and+BABA+RS plants at the end of the experimental period (3 weeks after inoculation) showed that pre-treatment with BABA generally enhanced the growth of the tomato plants compared to the inoculated control. Compared to -BABA+RS plants, the+BABA+RS treatment significantly increased FW of tomato shoots and roots by 49.9 and 41.9 %, respectively (Fig. 1a, b) whereas DW of tomato shoots was not significantly increased (Fig. 1c). Root DW was significantly increased by 35.6 % (Fig. 1d). In+BABA-RS plants, there was no significant difference in FW and DW of shoots and roots compared to the -BABA-RS plants (Fig. 1).

Effect of soil application of BABA on PPO and CAT activities

In general, the application of BABA resulted in the highest increase in PPO activity in both inoculated and non-inoculated plants (Fig. 2a). In+BABA+RS plants, there was a progressive and significant increase of PPO activity to reach a maximum level at the end of the experiment (8 days) and the enzyme activity was significantly higher than in plants receiving any of the three other treatments. PPO activity in -BABA+RS plants was significantly higher compared to -BABA-RS plants 4 days after inoculation (dai) and remained at the same level. On the other hand, there was no significant change of PPO level in -BABA-RS plants throughout the experimental period.

CAT activity in+BABA+RS plants decreased over time and became significantly lower than in -BABA+RS, +BABA-RS and -BABA-RS plants (Fig. 2b). Although CAT activity decreased in both -BABA+RS and+BABA-RS plants, the largest decrease of CAT activity occurred in+BABA+RS plants, with levels significantly lower than in the corresponding control (-BABA-RS). CAT levels decreased at 2 dai in -BABA+RS-treated tomato plants, but remained at the same level during last two time points. Both inoculated (+BABA+RS) and non-inoculated (+BABA-RS) tomato plants treated with BABA showed a significant decrease in CAT activities at 2 dai. After this time (4 days after treatment or 2 dai), CAT activity in+BABA+RS plants gradually and significantly decreased for the rest of experiment. Contrary to this, CAT level in+BABA-RS plants significantly increased.

Table 1 Effect of soil drenching with BABA on leaf wilting index (LWI) and vascular browning index (VBI) of bacterial wilt and bacterial population of *R. solanacearum* isolate (RS7) in tomato stem tissues under greenhouse conditions

Treatments	Severity of bacterial wilt		Bacterial population (cfu/g stem tissue)
	LWI	VBI	
-BABA+RS	53.9 a	55.8 a	4.3×10^{9} a
-BABA-RS	0.0 c	0.0 c	0.0 c
+BABA+RS	13.3 b	16.8 b	1.3×10^{7} b

Within each column, different letters indicate significant differences between treatments according to a least significant difference test (P=0.05)

Effect of soil application of BABA on free and total SA accumulation

Total SA levels (Fig. 3a) were about 5–8 fold higher than those of free SA (Fig. 3b). Application of BABA induced a significant increase of total SA in both+BABA-RS and+BABA+RS plants at all sampling times compared with untreated plants (Fig. 3a). In untreated plants, the inoculation with *R. solanacearum* significantly increased the total SA level in -BABA+RS plants compared with the control (-BABA-RS). In contrast, BABA treatment induced a rapid, significant, increase of total SA in+BABA-RS and+BABA+RS leaves compared to levels in untreated leaves (-BABA-RS and -BABA+RS). In+BABA+RS plants, total SA significantly increased until 4 days after treatment (2 dai) and remained at the same level during the rest of the experimental period.

BABA treatment generally increased the free SA level and it was significantly higher in+BABA+RS than in+BABA-RS plants (Fig. 3b). The lowest

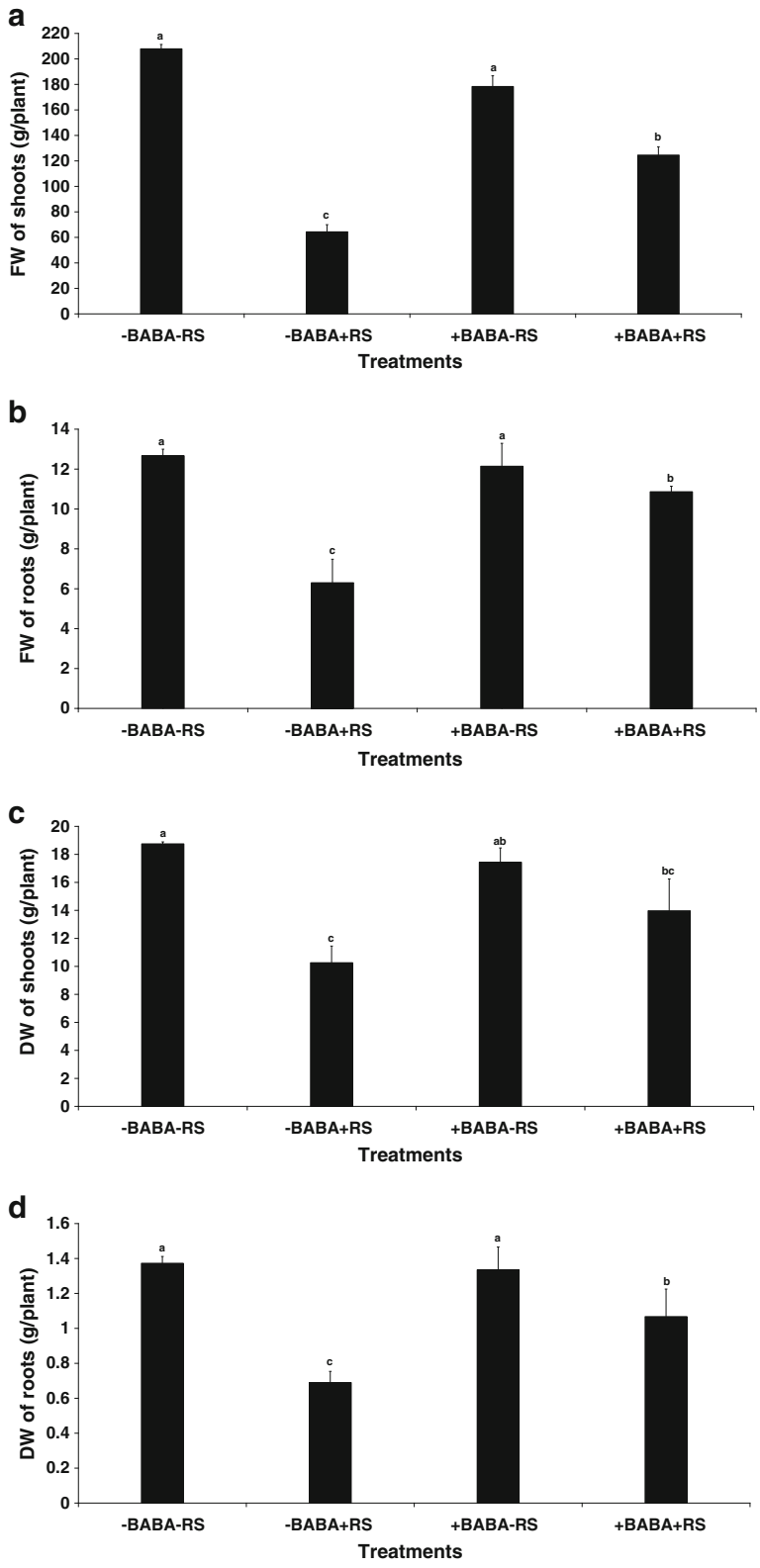


Fig. 1 Effect of soil drenching with BABA on fresh (FW) and dry weight (DW) of shoots and roots of tomato plants (g/plant). FW of shoots (**a**) and roots (**b**) and DW of shoots (**c**) and roots (**d**) were determined 3 weeks after inoculation with *R. solanacearum* (isolate RS7). Columns marked with the same letters are not significantly different according to Duncan's Multiple Range Test ($P<0.05$). *Vertical bars* represent± standard error (SE)

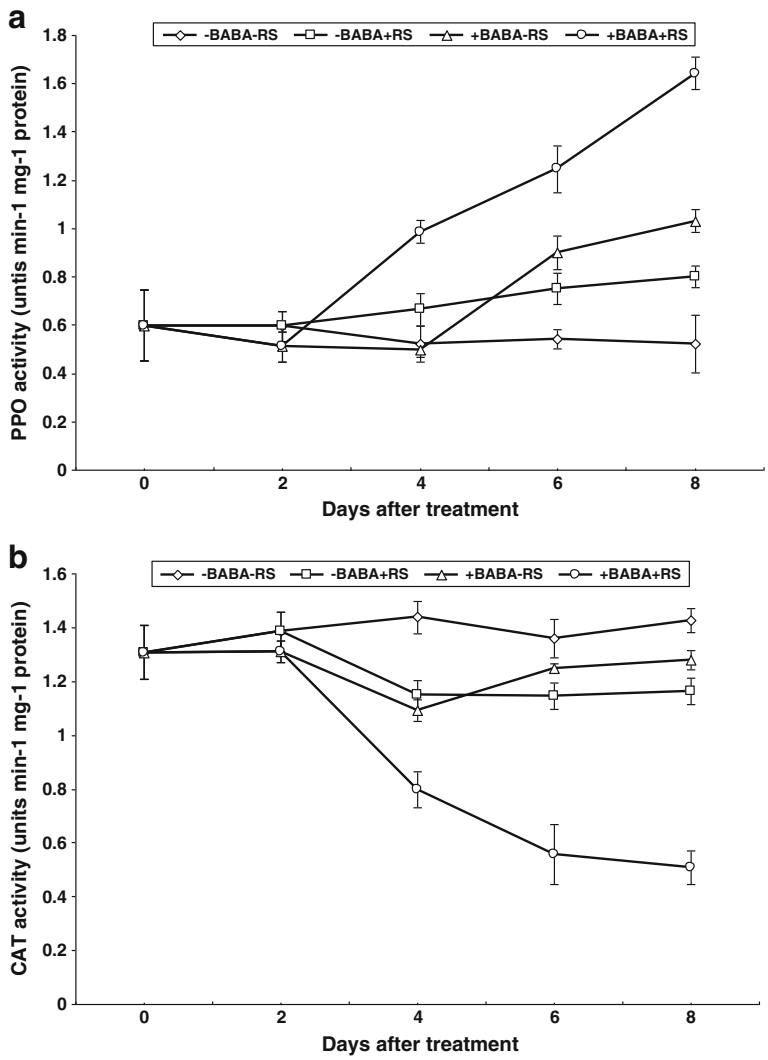


Fig. 2 Changes in the levels of (**a**) polyphenol oxidase (PPO) and (**b**) catalase (CAT) activities in leaves of tomato plants infected with *R. solanacearum* isolate (RS7). Tomato plants were inoculated 2 days after treatment with BABA or water. *Vertical bars* represent±standard error (SE)

amount of free SA was found in -BABA-RS plants. After inoculation with *R. solanacearum*, the free SA level increased gradually and significantly with time in+BABA+RS plants and reached a maximum at 8 days. Infection with *R. solanacearum* only significantly induced free SA in -BABA+RS plants after 4 dai compared to -BABA-RS plants.

Effect of soil application of BABA on total phenolic compounds

There was a significant increase in total phenolic compounds in+BABA+RS and+BABA-RS plants compared to the -BABA+RS and -BABA-RS plants (Fig. 4). There were no significant differences between -BABA+RS and -BABA-RS plants. Likewise, there were no significant differences between+BABA+RS and+BABA-RS plants.

Relationship between catalase activity and total salicylic acid

The decrease in CAT activity and its correlation to changes in the SA content were investigated in inoculated and non-inoculated tomato plants after BABA treatment (Fig. 5). In non-inoculated, water-treated plants (-BABA-RS, Fig. 5a), there was no significant change in CAT activity and total SA levels during the experiment. In BABA-treated plants (+BABA-RS, +BABA+RS, Fig. 5c-d), CAT activity significantly decreased at 2 dai, with the decrease being more pronounced with increasing total SA accumulation.

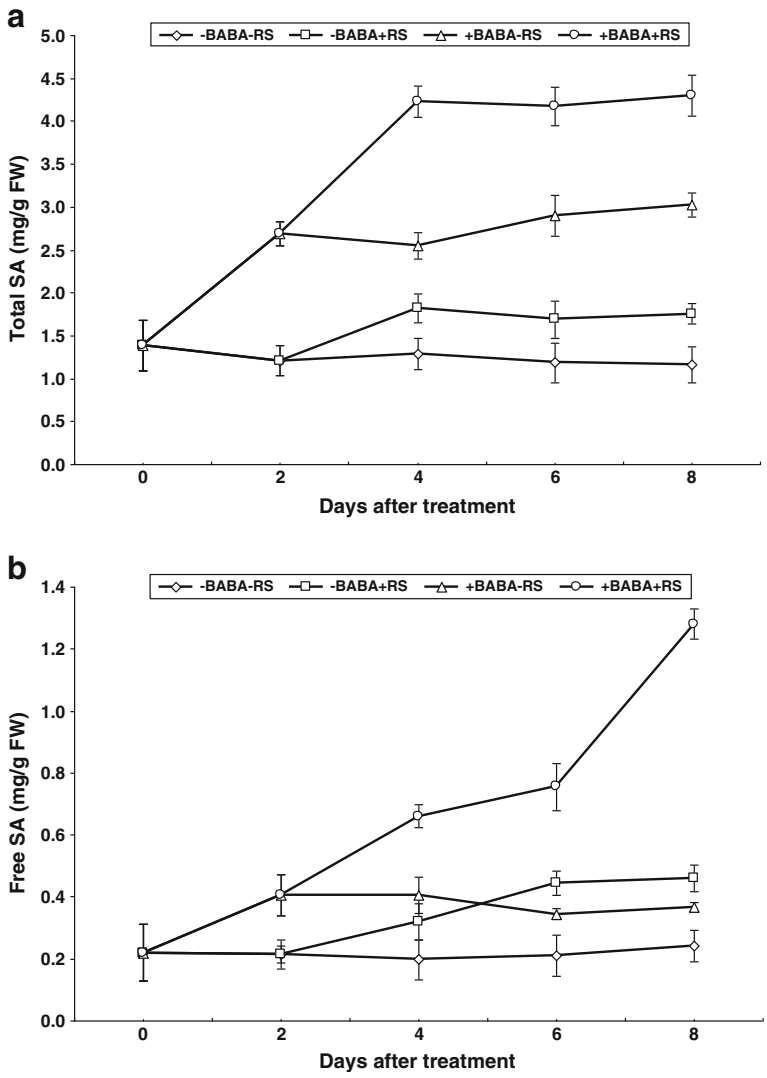


Fig. 3 Changes in the levels of (**a**) total and (**b**) free salicylic acid (SA) in leaves of tomato plants infected with *R. solanacearum* (isolate RS7). Tomato plants were inoculated 2 days after treatment with BABA or water. *Vertical bars* represent±standard error (SE)

Whereas the CAT activity continued decreasing in+BABA+RS plants during the rest of the experiment, it stabilised almost at the same level as before 2 dai in+BABA-RS plants. A significant, decrease in CAT activity with increasing SA concentration was observed in+BABA+RS and -BABA+RS plants at 2 dai (Fig. 5b and d). Later on, no significant change was found in CAT activity and total SA concentration in inoculated control plants (-BABA+RS).

Discussion

Here, we show that DL-3-aminobutyric acid (BABA) applied to the soil of greenhouse-grown tomato plants protected against bacterial wilt caused by *Ralstonia solanacearum*. The induction of phenolic compounds, salicylic acid and activities of selected plant defence enzymes were also observed. BABA is a non-protein amino acid known to induce resistance against a wide spectrum of pathogens (Jakab et al. 2001; Cohen 2002; Cohen et al. 2011). Although, some reports indicate that pre-treatment with BABA is effective against certain bacterial tomato diseases (Cohen 2002; Baysal et al. 2005), there is no information concerning the use of BABA against bacterial wilt of tomato caused by *R. solanacearum*. BABA has been reported to induce resistance against pathogens (Jakab et al. 2001; Zimmerli et al. 2001; Ton and Mauch-Mani 2004; Walters et al. 2005). It has also been

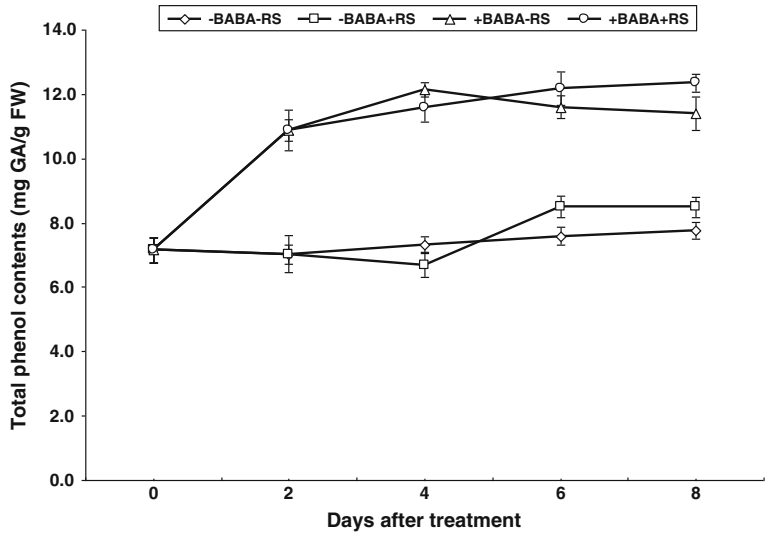


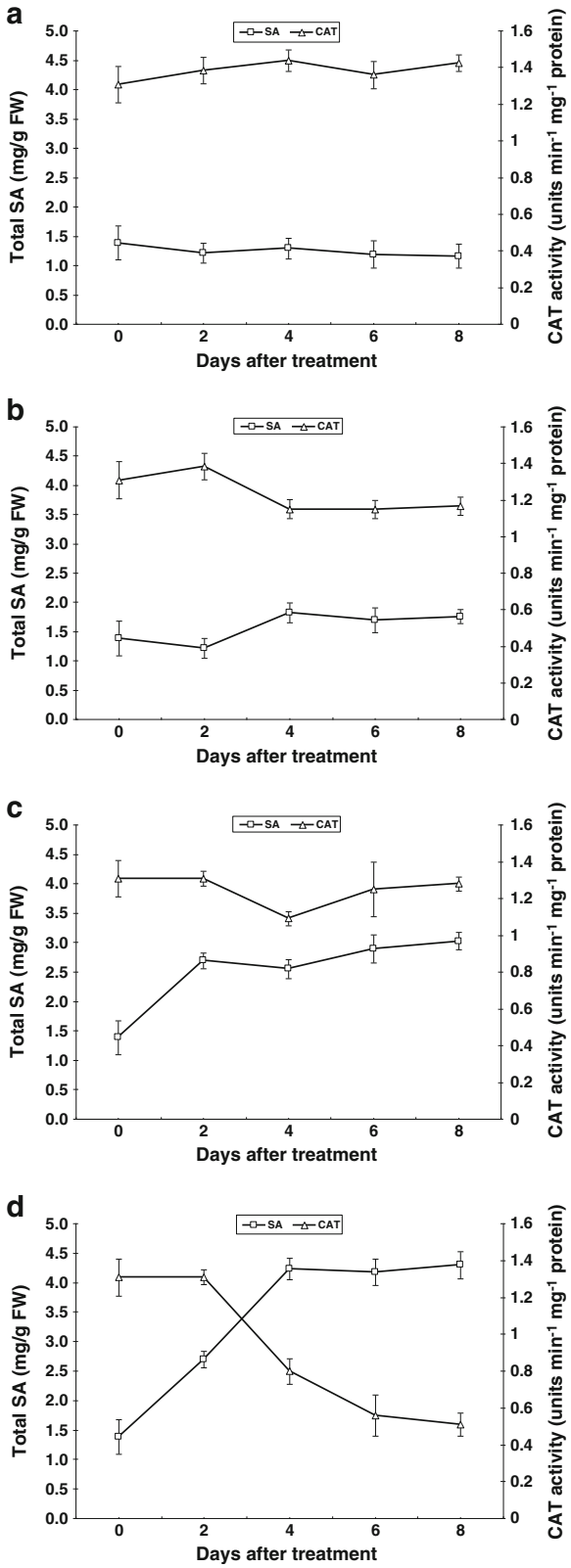
Fig. 4 Changes in the levels of total phenolic compounds in leaves of tomato plants infected with *R. solanacearum* (isolate RS7). Tomato plants were inoculated 2 days after treatment with BABA or water. *Vertical bars* represent±standard error (SE)

demonstrated that BABA operates via a variety of defence mechanisms including physical barriers and biochemical changes including the hypersensitivity response and accumulation of callose and lignin (Ton and Mauch-Mani 2004; Walters et al. 2005). The study here indicates that the reduction in bacterial wilt disease severity is associated with reduced titres of *R. solanacearum* in BABA treated plants. We suggest that bacterial growth is inhibited by toxic substances formed by the tomato plant after application of BABA. It may be assumed that reduction of bacterial multiplication in+BABA+RS plants was accompanied by accumulation of defence constituents in plant tissue especially in the xylem. Antimicrobial compounds, for example acidic PR-proteins, phenolic acids, peroxidases, lignin and other defence compounds may accumulate in plant tissue treated with BABA (Walters et al. 2005).

BABA treatment not only reduced the population of *R. solanacearum* in+BABA+RS plants, but also enhanced growth of tomato plants compared to inoculated control (-BABA+RS) plants. Although FW and DW of shoots and roots of+BABA+RS plants did not increase compared to non-inoculated control (-BABA-RS) plants, they were significantly higher than those of -BABA+RS plants. This may be due to reduction of the disease incidence in -BABA+RS plants in addition to an increase of vegetative characters in+BABA+RS plants. It could be hypothesized that the hormonal balance of the plants changed during induction of resistance or the effect could be due to interactions of a family of hormones. Thus, induction of resistance against downy mildew caused by *Sclerospora graminicola* by BABA application enhanced vegetative growth, i.e., height, fresh weight, leaf area and tillering of pearl millet and in these plants, the hormonal balance was found to be altered by BABA treatment (Shailasree et al. 2001).

In this study we focused on the possible role of PPO and CAT in protection of tomato against the bacterial wilt pathogen *R. solanacearum*. BABA enhanced PPO activity in both inoculated and non-inoculated plants. PPO is a copper-containing enzyme known to be involved in resistance against *R. solanacearum* in resistant tomato cultivars (Vanitha et al. 2009). PPO is important in defence against pathogens through its role in oxidation of phenolic compounds into antimicrobial quinines (Barilli et al. 2010). We also found that BABA significantly increased total phenolics in both inoculated and non-inoculated tomato plants compared to water-treated control plants. Accumulation of phenolic compounds at the infection site has been correlated with the restriction of pathogen development due to their toxic character. Moreover, resistance may be increased by a change in pH in the plant cell cytoplasm due to the increase in phenolic acid

Fig. 5 Activity of catalase (CAT) after application of β-aminobutyric acid (BABA) and relationship to total salicylic acid (SA) accumulation in leaves of tomato plants. Tomato plants were inoculated with *R. solanacearum* (isolate RS7) 2 days after treatment with BABA or water. (**a**) non-inoculated control plants (-BABA-RS); (**b**) inoculated control plants (-BABA+RS); (**c**) non-inoculated treated plants (+BABA-RS) and (**d**) inoculated treated plants (+BABA+RS) ▶



content, thus resulting in inhibition of pathogen development (Nicholson and Hammerschmidt 1992). Our data corroborate with other reports showing that BABA induced accumulation of phenolic compounds and PR-proteins in tomato roots (Chamsai et al. 2004) and improved resistance to *Uromyces pisi* in pea plants by enhancing the total phenolic content (Barilli et al. 2010).

Contrary to PPO activity, Catalase (CAT) activity decreased in+BABA+RS plants compared with -BABA-RS and+BABA-RS plants. Catalases are ubiquitous in living organisms, and play a role in plant defence against pathogens. Catalase inhibition would lead to hydrogen peroxide (H_2O_2) accumulation (Bhattacharjee, 2005). Higher concentrations of H_2O_2 in resistant than in susceptible tomato cultivars have been reported in several plant pathogen interactions (Mandal et al. 2011). H_2O_2 is believed to play different roles in defence, i.e., restriction of pathogen growth (e.g., for *Erwinia carotovora* ssp. *carotovora* and *Phytophthora infestans*) and induction of phytoalexins and PR-proteins (Bhattacharjee, 2005). H_2O_2 also play an essential role in lignification, and cross-linking of cell wall proteins with phenolic acids and leads to a reinforcement of cell walls at the site of pathogen attack. Cohen et al. (2011) showed that H_2O_2 accumulation in lettuce plants treated with BABA protected against *Bremia lactucae*. Furthermore, Shetty et al. (2008) discussed the different roles of ROS and H_2O_2 in host-pathogen interactions (wheat-*Septoria tritici*/*Blumeria graminis* f.sp. *tritici*) and particularly that H_2O_2 plays an important role in defence, by driving, among others, cell wall cross-linking to strengthen the cell wall. Furthermore, elevated levels of H_2O_2 has also been shown to activate the expression of several defence genes and the plants showed enhanced resistance to rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* and the rice blast pathogen *Magnaporthe grisea* (Kachroo et al. 2003). The restriction of *R. solanacearum* growth could be due to the antimicrobial activity of hydrogen peroxide, which is strongly increased around bacterial cells, and to the oxidative cross-linking of the cell wall, driven by the rapid accumulation of H_2O_2 at the plant cell walls adjacent to attached bacteria (Buonaurio, 2008). Moreover, *R. solanacearum* was affected in growth and disease development in tobacco plants that overproduce H_2O_2 (Flores-Cruz and Allen 2009), further strengthening the evidence that host-produced ROS (H_2O_2) can limit pathogen success. It is assumed that H_2O_2 accumulation results in antimicrobial

activity through strengthening of the plant cell wall, activation of defence genes, hypersensitive cell death and a subsequent halt of *R. solanacearum* infection.

Generation of reactive oxygen species (ROS), especially H_2O_2, in plants in response to pathogen attack and the mechanisms by which ROS enhance the resistance through ROS detoxifying enzymes have been recently investigated (Colburn-Clifford et al. 2010; Flores-Cruz and Allen 2009; Shetty et al. 2008). Based on this, we hypothesise that the accumulation of ROS could lead to activation of defence genes coding for PR-proteins, enzymes involved in the generation of phytoalexins, enzymes involved in protection against oxidative stress, lignification and other defence responses in+BABA+RS plants. Flores-Cruz and Allen (2009) found that *R. solanacearum* was affected in growth and disease development in tobacco plants that overproduce H_2O_2, suggesting that host-produced ROS can limit pathogen infection. In addition, it can be speculated whether the increased level of H_2O_2 production, combined with a more efficient antioxidant system and high lignin deposition in the cell wall, may contribute to the resistance of tomato plants to *R. solanacearum*.

Salicylic acid (SA) has been shown to be an important signalling molecule involved in defence responses to pathogen attack in many plant-pathogen interactions (Shetty et al. 2008). In the present study, BABA treatment induced a significant increase of free and total SA in+BABA+RS and+BABA-RS plants at all sampling times compared with control plants. The study by Milling et al. (2011) and Chen et al. (2009) suggested that tomato fends off the bacterial wilt by multiple defence mechanisms, which involve ET- and SA-related defence signalling pathways. Probably, increases in the free and total levels of SA in+BABA+RS plants leads to the elevated expression of genes encoding the pathogenesis-related (PR) proteins and the activation of disease resistance, so that *R. solanacearum* is confronted with several defence responses at the time of infection. On the basis of our results, we therefore propose that the resistance of tomato to bacterial wilt involves SA-related signalling pathways.

Although the content of total SA increased in tomato plants treated with BABA, it was inversely correlated with the decrease in the CAT activity. Our results agree with previous results (Sawada et al. 2006; Shim et al. 2003) which suggested that the decrease in CAT activity is a phenomenon occurring in many plant species under oxidative stress and is related to the accumulation of SA in oxidatively-stressed plants. So, we propose that one mode of action of SA could be its inhibition of CAT activity. In accordance with the explanation of Chen et al. (1995), we believe that CAT can be considered a receptor for SA. It perceives the SA signal by binding this ligand and then transduces this signal by inhibiting its own enzymatic activity, which leads to elevated H_2O_2 levels. The inhibition of CAT activity has been discussed in relation to induced resistance to plant pathogens (Shim et al. 2003) and it has been reported that SA may be the compound causing inhibition of CAT and induction of disease resistance by the signalling of H_2O_2 and activation of the defence genes (Mehdy 1994).

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