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Research article

Salicylic acid-induced resistance to *Fusarium oxysporum* f. sp. *lycopersici* in tomato

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ABSTRACT

We demonstrated that exogenous application of 200 μM salicylic acid through root feeding and foliar spray could induce resistance against *Fusarium oxysporum* f. sp. *Lycopersici* (*Fol*) in tomato. Endogenous accumulation of free salicylic acid in tomato roots was detected by HPLC and identification was confirmed by LC–MS/MS analysis. At 168 h of salicylic acid treatment through roots, the endogenous salicylic acid level in the roots increased to 1477 ng g^{-1} FW which was 10 times higher than control plants. Similarly, the salicylic acid content was 1001 ng g^{-1} FW at 168 h of treatment by foliar spray, which was 8.7 times higher than control plants. The activities of phenylalanine ammonia lyase (PAL, EC 4.3.1.5) and peroxidase (POD, EC 1.11.1.7) were 5.9 and 4.7 times higher, respectively than the control plants at 168 h of salicylic acid feeding through the roots. The increase in PAL and POD activities was 3.7 and 3.3 times higher, respectively at 168 h of salicylic acid treatments through foliar spray than control plants. The salicylic acid-treated tomato plants challenged with *Fol* exhibited significantly reduced vascular browning and leaf yellowing wilting. The mycelial growth of *Fol* was not significantly affected by salicylic acid. Significant increase in basal level of salicylic acid in noninoculated plants indicated that tomato root system might have the capacity to assimilate and distribute salicylic acid throughout the plant. The results indicated that the induced resistance observed in tomato against *Fol* might be a case of salicylic acid-dependent systemic acquired resistance.

1. Introduction

Fusarium wilt caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) is one of the most devastating diseases of tomato. The disease causes great losses, especially on susceptible varieties of tomato and when soil and air temperatures are rather high during much of the season such as in warm climates [1]. The classical strategies, viz., use of fungicides and resistant varieties have largely been ineffective in controlling the disease because of soil-borne nature and emergence of new race of the pathogen. Furthermore, there are increasingly more restrictions on application of fungicides due to public concern about residues in food and harmful effects on the environment and human health. Hence there is a need for developing novel management strategy which is practically effective and environmentally safe.

Induction of resistance to pathogen is a promising approach for controlling plant diseases. Induced resistance is the general term by which all types of elicited responses that lead to enhanced protection against disease – including both locally and systemically induced resistance – can be designated [2]. One of the classic forms of induced resistance is systemic acquired resistance (SAR) controlled by a signaling pathway that depends on endogenous accumulation of salicylic acid (SA) [3].

Exogenous or endogenous factors could substantially affect host physiology, leading to rapid and coordinated defense-gene activation in plants normally expressing susceptibility to pathogen infection. Chitosan treatment induced a significant increase in the activities of polyphenoloxidase, peroxidase, and enhanced the content of phenolic compounds in tomato fruits, thus providing protection against gray mould and blue mould diseases [4]. Plant resistance can be induced by application of synthetic compounds such as functional analogs of SA, e.g. benzothiadiazole-7-carbothioic acid (acibenzolar-S-methyl) or BTH. It has been shown that BTH induced systemic resistance by root-treatment in

Abbreviations: Fol, *Fusarium oxysporum* f. sp. *lycopersici*; FW, Fresh weight; PAL, Phenylalanine ammonia lyase (EC 4.3.1.5); POD, Peroxidase (EC 1.11.1.7); SA, Salicylic acid; SAR, Systemic acquired resistance.

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tomato and controlled crown and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici* [5]. *Fusarium* wilt of tomato was effectively controlled by foliar spray of validamycin A or validoxylamine A, which induced SA accumulation and development of systemic resistance [6]. Exogenous application of SA induces plant resistance to different kinds of pathogens that are associated with oxidative burst, cell wall enforcement, up- or down-regulation of gene expression [7].

Phenylalanine ammonia lyase (PAL) catalyzes the deamination of L-phenylalanine to *t*-cinnamic acid, which is the first step in the phenylpropanoid pathway which supplies the precursors for phenolics, lignin and furanocoumarin, phytoalexins and other downstream metabolites [8]. Peroxidase (POD) oxidizes phenolics to more toxic quinones and generates hydrogen peroxide. The last step in the synthesis of lignin and suberin has been proposed to be catalyzed by peroxidases [9]. The activities of PAL and POD may rapidly be enhanced under the influence of elicitors or pathogen attack. Enhancement of PAL and POD activities was reported in response to *Rhizoctonia solani* inoculation in cowpea pretreated with SA [10]. PAL exhibits high reactivity to stress factors and plays a key role in the synthesis of compounds involved in phytoimmunity. Latunde-Dada and Lucas [11] showed acibenzolar-S-methyl mediated systemic priming of phenylalanine ammonia lyase (PAL) in cowpea seedlings and enhanced resistance against the causal agent of anthracnose *Colletotrichum destructivum*. The POD activity was induced in *Cucurbita pepo* leaves in response to zucchini yellow mosaic virus infection and salicylic acid treatments [12].

The objective of the present work was to investigate if exogenous application of SA can induce resistance in tomato that is effective against *Fusarium* wilt. *Fol* is a soil-borne pathogen and infects the plants systemically through roots. Hence, hydroponically grown tomato plants were treated with SA by root feeding and foliar spray and then challenged with *Fol*. Root tissues were analysed to measure the endogenous levels of free SA and root response to pathogen infection.

2. Results

2.1. Detection and identification of SA in the roots of tomato

Free SA from the roots of tomato was detected and identified by HPLC and LC–MS/MS. The endogenous free SA in tomato roots, after the plants were fed exogenously with SA through root feeding and foliar spray, was detected and identified by an isocratic HPLC method (Fig. 1). Identification of HPLC detected SA was further confirmed by highly reliable LC–MS/MS technique (Fig. 2). Samples from the roots of tomato plants treated with salicylic acid through foliar spray were analysed by LC–MS/MS in comparison to authentic standard. The LC–MS/MS chromatograms of HPLC detected samples confirmed the identification of SA in the roots of tomato plants.

2.2. Effect of root feeding and foliar spray of SA on endogenous level of SA in the tomato roots challenged with Fol

Hydroponically grown tomato plants were fed with 200 μM SA through roots and leaves for 7 days, and then were challenged with *Fol* after two days (i.e. 48 h) of last SA application. The addition of 200 μM SA directly to the root system significantly increased the endogenous free SA content of the roots (Fig. 3a). The increase in free SA content in roots of the treated plants was noticed even after 24 h of SA feeding through roots. Free SA level of the treated tomato roots was 862.5 ng g^{-1} FW after 72 h of treatment, which was approx. 8.5 times higher than the basal level of SA. This level of SA had no visible phytotoxic effects on the plants. At 168 h of SA treatment, the endogenous free SA level in the root tissues increased to 1477 ng g^{-1} FW which was 10 times higher as compared to the control plants.

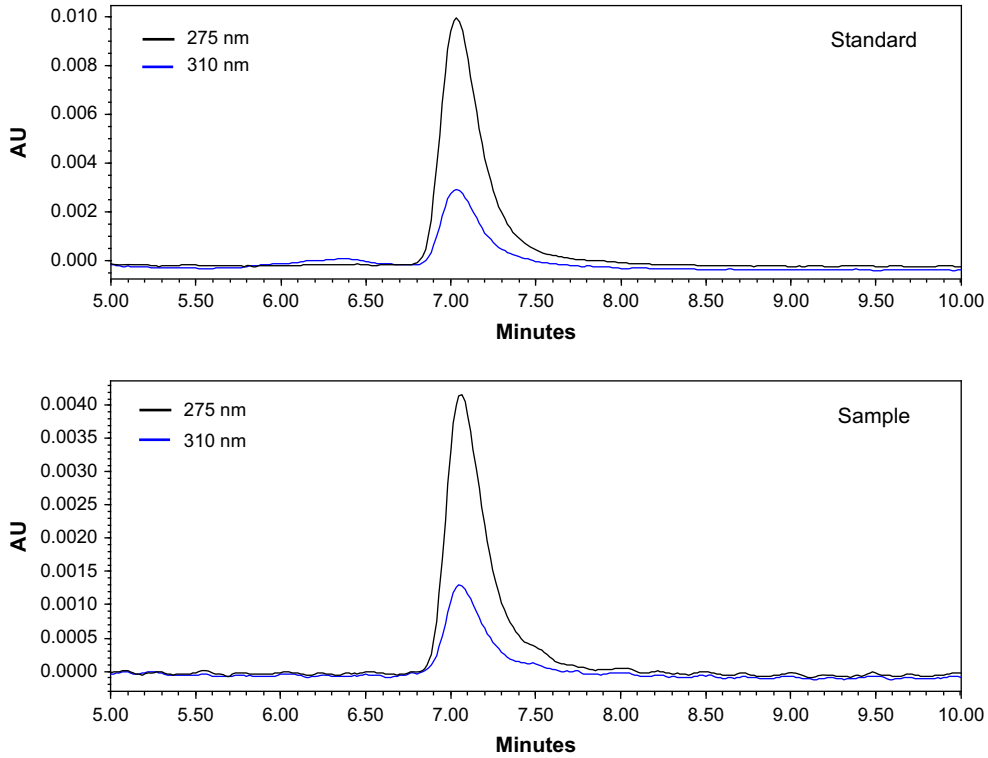

Fig. 1. Overlay of HPLC chromatograms showing accumulation of salicylic acid (SA) detected at 275 nm and 310 nm in roots of tomato treated with salicylic acid through foliar spray. See Materials and methods for technical details of SA extraction and HPLC analysis.

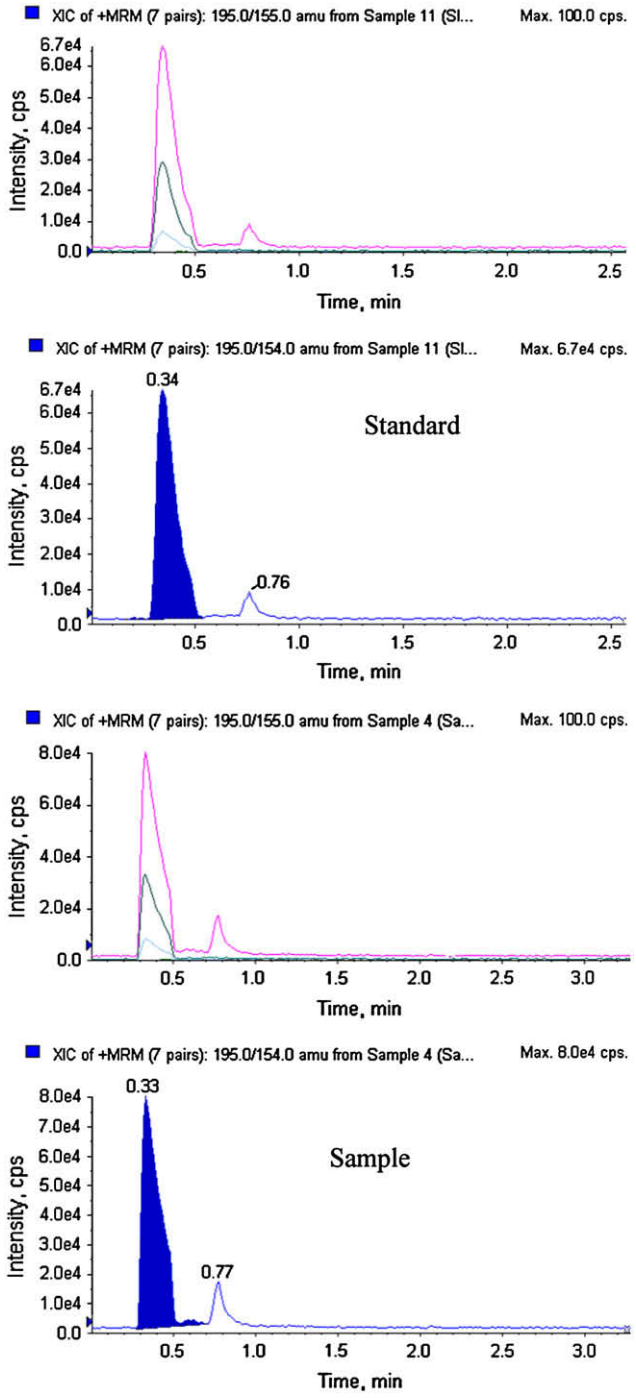

Fig. 2. LC–MS/MS chromatograms of standard salicylic acid and that from the roots of tomato (sample) treated with salicylic acid through foliar spray. See Materials and methods for technical details of LC–MS/MS analysis.

It was observed that spraying of 200 μM SA on the leaves of tomato plants could increase the free SA level in the distant systemic root tissues of the plants to a significant extent (Fig. 3b). Like root feeding, the increase in free SA content in roots of the treated plants was noticed from the first time point of analysis, i.e. 24 h after SA application on the leaves. Free SA content in the roots was 542.5 ng g^{-1} FW (4.5 times higher) than the control plants at 72 h of treatment. In a very similar manner, the free SA content was 1001 ng g^{-1} FW at 168 h of foliar treatment, and it was 8.7 times higher than the control plants.

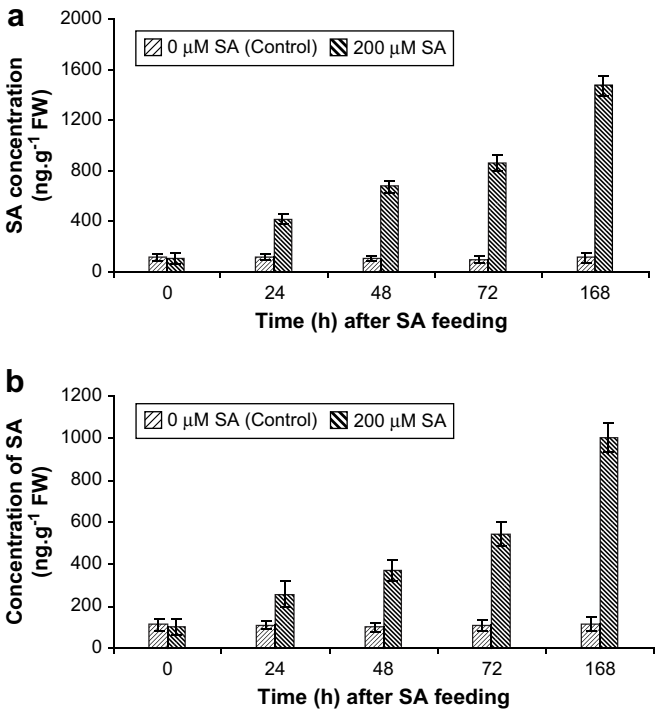

Fig. 3. Accumulation of free salicylic acid (SA) in roots of tomato plants treated with SA through root feeding (a) and foliar spray (b). Hydroponically grown tomato plants were treated with SA (0 μM and 200 μM) through root feeding and foliar spray for 7 days. The plants were inoculated with *Fusarium oxysporum* f. sp. *lycopersici* after two days (i.e. 48 h) of last SA application. Roots were harvested at 0, 24, 48, 72 and 168 h after SA treatment and analysed for determination of free SA by HPLC. Data bars are mean ± SD of three independent experiments with three replicates.

2.3. SA treatment of tomato plants potentiates PAL and POD activities in the roots of tomato plants upon Fol infection

Activity of the defense enzyme PAL was observed to increase sharply in response to root feeding and foliar spray of SA. PAL activity was 3.5 times higher than control at 72 h after 200 μM SA feeding of tomato plants through the roots than the control plants. The activity of the enzyme was 5.9 times higher than the control plants on day seven (i.e. 168 h) of the SA feeding of the roots (Fig. 4a). Similarly, PAL activity was 2.9 times higher than control at 72 h after 200 μM SA feeding of tomato plants through foliar spray than the control plants. The activity of the enzyme was 3.7 times higher than the control plants on day seven (i.e. 168 h) of the SA feeding of the leaves (Fig. 4b).

Similarly, POD activity showed a sharp increase in response to root feeding and foliar spray of 200 μM SA. POD activity was 4 times higher than control at 72 h after 200 μM SA feeding of tomato plants through the roots than the control plants. The activity of the enzyme was 4.7 times higher than the control plants on day seven (i.e. 168 h) of the SA feeding of the roots (Fig. 5a). POD activity was 2.5 times higher than control at 72 h after 200 μM SA feeding of tomato plants through foliar spray than the control plants. The activity of the enzyme was 3.3 times higher than the control plants on day seven (i.e. on 168 h) of SA feeding of the leaves (Fig. 5b).

2.4. SA treatment of tomato plants induces resistance against Fol infection

Tomato plants grown hydroponically were exogenously fed with SA through roots and leaves, and then challenged with *Fol* after two days, i.e. 48 h of last SA application. The addition of 200 μM SA to

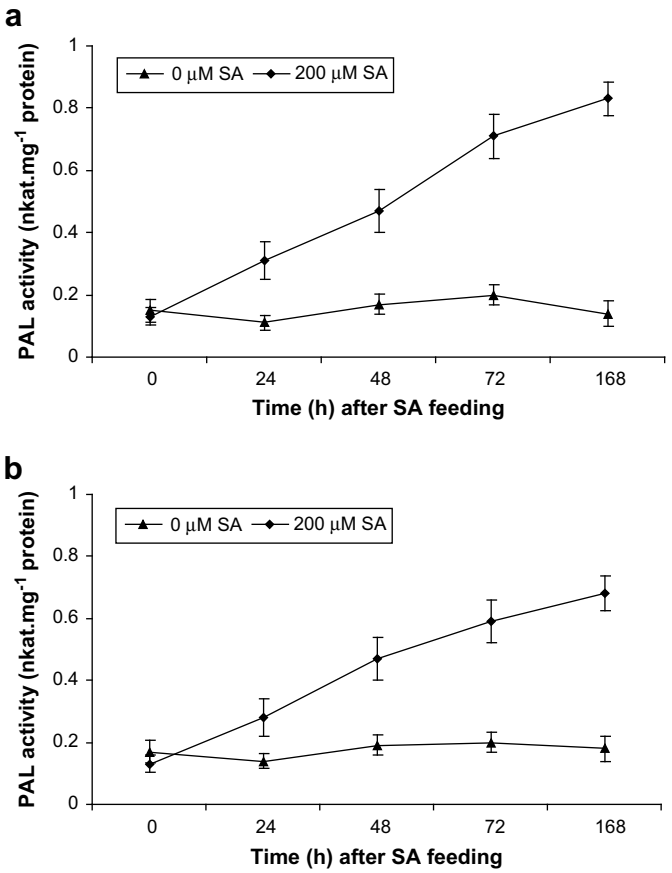

Fig. 4. Phenylalanine ammonia lyase (PAL) activity in roots of tomato after the plants were treated with 200 μM salicylic acid (SA) through root feeding (a) and foliar spray (b). Hydroponically grown tomato plants were treated with SA (0 μM and 200 μM) through root feeding and foliar spray for 7 days. The plants were inoculated with *Fusarium oxysporum* f. sp. *lycopersici* in the roots after two days (i.e. 48 h) of last SA application. Roots were harvested at 0, 24, 48, 72 and 168 h after SA treatment and PAL activity was assayed. Data bars are mean ± SD of three independent experiments with three replicates.

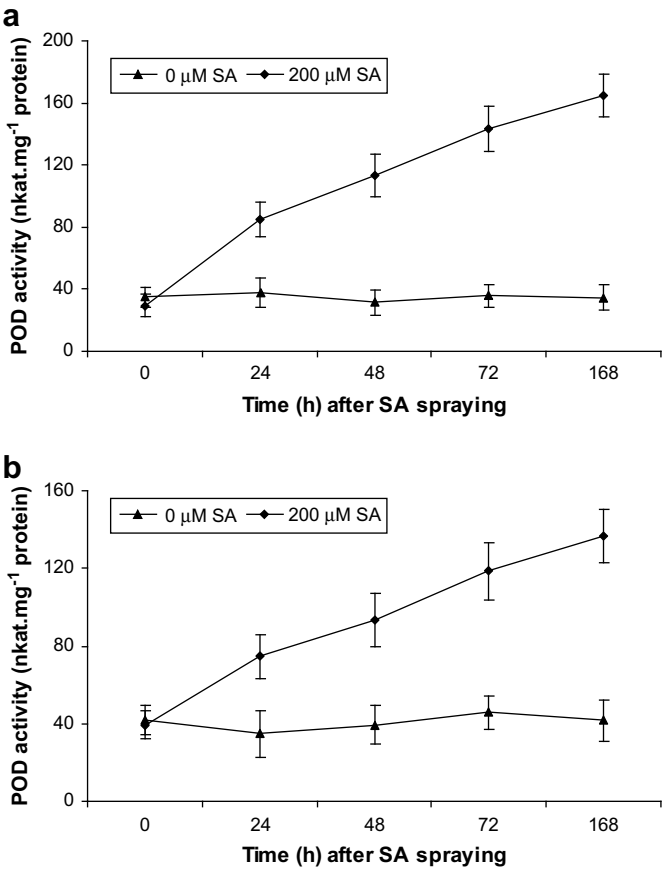

Fig. 5. Peroxidase (POD) activity in roots of tomato after the plants were treated with 200 μM salicylic acid (SA) through root feeding (5a) and foliar spray (b). Hydroponically grown tomato plants were treated with SA (0 μM and 200 μM) through root feeding and foliar spray for 7 days. The plants were inoculated with *Fusarium oxysporum* f. sp. *lycopersici* in the roots after two days (i.e. 48 h) of last SA application. Roots were harvested at 0, 24, 48, 72 and 168 h after SA treatment and POD activity was assayed. Data bars are mean ± SD of three independent experiments with three replicates.

the hydroponics medium significantly affected infection and wilt development by *Fol* on tomato plants. The percent of vascular browning and leaf yellowing wilting was markedly reduced when plants were grown in presence of 200 μM SA (Fig. 6). Tomato plants inoculated with *Fol* conidia, but not receiving 200 μM SA treatment through roots, exhibited typical vascular browning and leaf yellowing wilting, while the SA-treated plants showed less than 25% vascular browning and leaf yellowing wilting after 4 weeks of the experiment (Fig. 7a).

Similarly, the foliar application of 200 μM SA on the hydroponically grown tomato plants significantly affected infection and wilt development by *Fol* on tomato plants. The tomato plants inoculated with *Fol* conidia, but not receiving 200 μM SA treatment as foliar spray, exhibited characteristic vascular browning and leaf yellowing wilting, while the SA-treated plants showed less than or equal to 25% vascular browning and leaf yellowing wilting after 4 weeks of the experiment (Fig. 7b).

It was observed that the inoculated plants not fed with 200 μM SA did not accumulate higher level of free SA. In other words, the basal level of free SA did not significantly change in response to infection by *Fol* without pretreatment with 200 μM SA. Whereas the tomato plants treated with 200 μM SA previously and then inoculated with *Fol* were found to accumulate more than 2 times free SA in the roots than the noninoculated plants (Fig. 8).

2.5. In vitro antifungal activity assay of SA against Fol

The mycelial growth of *Fol* was not significantly affected by SA amendment of the PDA culture medium (Fig. 9). None of the three concentrations of SA tested, viz., 100 μM, 200 μM and 300 μM were found to inhibit mycelial growth of *Fol* significantly as compared to control.

3. Discussion

In the present study, we demonstrated that exogenous application of SA through root feeding and foliar spray can induce resistance in tomato against the soil-borne pathogen *Fol*. Induction of resistance was achieved by providing 200 μM SA directly to the root system of the plant and also by foliar spray of SA. Increased levels of SA in roots of tomato plants in response to root and leaf feeding of 200 μM SA were detected and identified by HPLC and LC–MS/MS analyses.

SA is well known for its endogenous signal molecule playing an important role in development of systemic resistance in plants [13]. Exogenous application of 200 μM SA to tomato plants through hydroponics medium could significantly elevate foliar SA levels and activate systemic resistance that was effective against *Alternaria solani* [14]. Recently, it has been shown that exogenous SA treatment prior to inoculation provided increased *F. oxysporum*

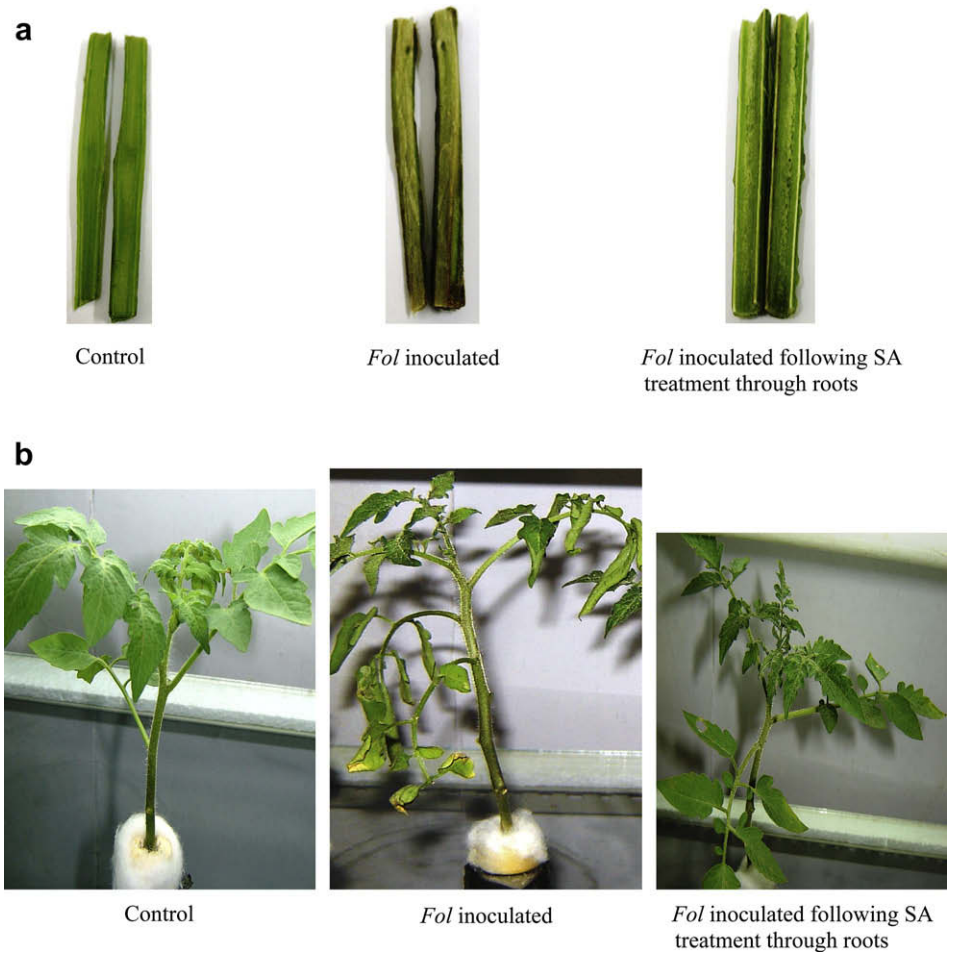

Fig. 6. Effects of 200 μM salicylic acid (SA) treatments and inoculation of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) on hydroponically grown tomato. (a) Vascular browning; (b) Leaf yellowing wilting.

resistance as evidenced by reduced foliar necrosis and plant death in *Arabidopsis* [15]. In our study, endogenous SA increased several fold in the roots of tomato due to exogenous SA treatment through root feeding and foliar spray prior to inoculation of *Fol*. This increased SA concentration in the root tissues might have contributed for enhanced resistance to the pathogen as was evidenced by significantly reduced vascular browning and leaf yellowing. Exogenous SA stimulated the systemic resistance against *Fusarium* wilt of chickpea and reduced the disease severity significantly [16]. It was found in this study that root and leaf feeding of SA to tomato plants resulted in substantial increase in the basal level of SA in noninoculated roots. The root system of tomato might have the capacity to assimilate SA, distribute the compound throughout the plant, and ultimately activate systemic disease resistance [14]. A similar response was observed in tobacco plants treated with 100 μM SA [7]. Role of SA has been proved to contribute to basal defense of tomato against *Botrytis cinerea* as well [17]. Potentiated responses induced by pathogen and elicitor have been observed in many plant species pretreated with SA and its functional analogs BTH and INA [18], and β-aminobutyric acid [19]. *F oxysporum* was shown to induce SAR and pathogenesis-related (PR) proteins in *Arabidopsis*, indicating that the SA pathway plays a role in plant resistance to *F. oxysporum*. Defense pathways mediated by SA and others seem to play an essential function in the modulation and networking of *Arabidopsis* innate immune response [20]. SA application induces accumulation of PR proteins [21]. Many of the PR proteins have antimicrobial activity *in vitro* and they serve as molecular markers for the onset of the defense response [3]. Application of exogenous SA and its functional analogs potentiated plant tissues to respond rapidly and effectively with a variety of defense mechanisms after pathogen challenge or elicitation [22]. *NahG*-transgenic plants and *Arabidopsis* mutants impaired in SA production exhibited enhanced susceptibility to a variety of pathogens, and demonstrated the importance of SA for SAR [23]. Pretreatment of asparagus roots with SA primed plants for a potentiated defense response to *F. oxysporum* f. sp. *asparagi* [24]. *In vitro* tests showed that SA had no direct antifungal effect on mycelial growth of *Fol* on PDA. This result proves that the role of SA in the plant is to activate the SA pathway and to induce finally antimicrobial peptides such as PR-1 which will affect directly to the fungal growth. He and Wolyn [25] observed that SA did not possess direct antifungal activity against *F. oxysporum* f. sp. *asparagi*, and disease resistance in asparagus was the result of plant defense mechanisms rather than direct inhibitory effects of SA on the fungus. This strengthens the hypothesis that SA activates the signal transduction pathway, thus leading to expression of SAR, rather than inhibiting the fungus directly [26]. However, it was also reported that salicylic acid as an allelochemical greatly inhibited *F. oxysporum* f. sp. *niveum* growth and conidia formation and germination, though stimulated mycotoxin production and activities of hydrolytic enzymes by *F. oxysporum* f. sp. *niveum* [27]. Hence, it may be useful to quantify the fungus by QRT-PCR specific fungal probes

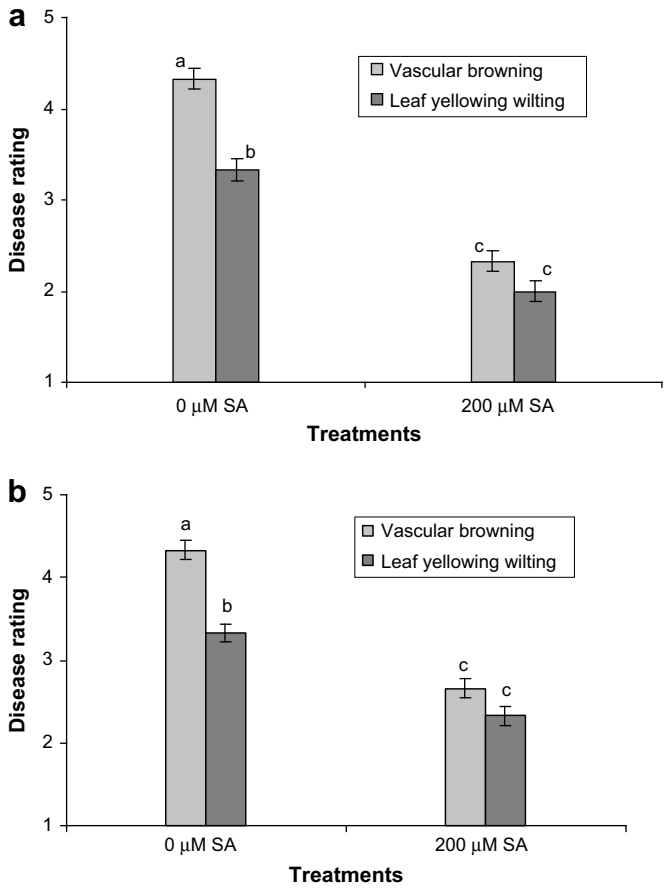

Fig. 7. Induction of resistance in tomato plants against *Fusarium oxysporum* f. sp. *lycopersici* infection by root feeding (a) and foliar spray (b) with 200 μM salicylic acid. Data on vascular browning of roots and leaf yellowing wilting were recorded 4 weeks after challenge of tomato plants by *Fol*. Columns represent the mean disease rating on a 1–5 scale as described in Materials and methods section. Columns with different letters are significantly different according to Student's *t*-test ($P < 0.05$). The experiments were repeated three times with three replicates.

or at least loss of weight of the plant, demonstrating clearly if growth of the fungus is affected at the plant after SA treatment or not.

PAL plays a key role in the synthesis of compounds involved in phytoimmunity. Many authors reported that PAL may serve as

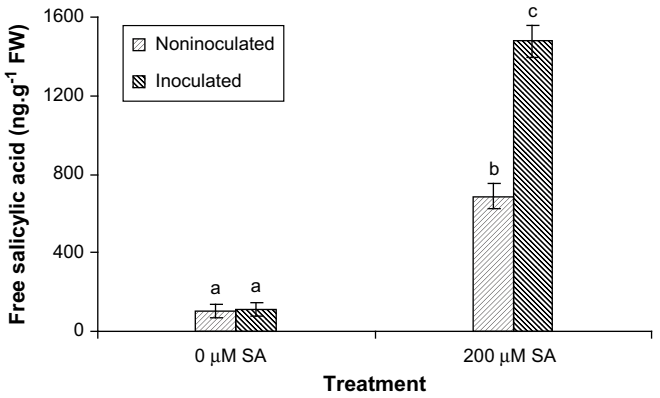

Fig. 8. Levels of free salicylic acid (SA) in noninoculated roots and in *Fusarium oxysporum* f. sp. *lycopersici* infected roots 120 h after inoculation with spore suspension. Data are the means ± SD of three independent experiments with three replicates in each experiment. Columns with different letters indicate a significant difference between noninoculated and infected plants according to Student's *t*-test ($P < 0.05$).

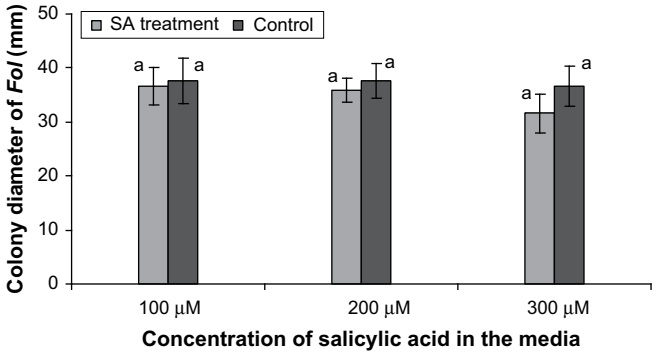

Fig. 9. Effect of salicylic acid (SA) on mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* culture. Fungal colonies were reared on potato dextrose agar amended with 100, 200, 300 μM SA and the colony diameters were measured on 9th day after inoculation of the fungus. Columns with same letters do not indicate a significant difference between the treatments according to Student's *t*-test ($P < 0.05$). Data bars are the means ± SD of four replicates.

a marker for induced resistance of plants to diseases [28]. PAL activity in plant tissue may rapidly change under the influence of various factors, e.g. pathogen attack and treatment with elicitors. PAL and POD activities were enhanced several fold in tomato roots by a biotic elicitor *Fusarium* mycelium extract derived from *Fol* [29]. Enhancement of PAL and POD activities in SA-treated asparagus plants upon *F. oxysporum* f. sp. *asparagi* infection resulted in the reinforcement of the cell wall and restricted subsequent fungal penetration and infection [25]. Addition of 20 μM salicylic acid to *Saussurea medusa* cell cultures resulted in 7.5-fold increase in PAL activity [30]. SA spraying on *Ya Li* pear plants increased PAL and POD activities greatly and contributed in protection of pear fruits against postharvest diseases [31]. In the present investigation also, the activities of PAL and POD increased to a great extent in the SA-treated plants compared to non-SA-treated plants, probably contributing in enhanced resistance of tomato to *Fol*.

It may not be out of place to mention that systemic resistance can also be induced against pathogenic *Fusarium* species by non-pathogenic *Fusarium* strains. A *Fusarium solani* strain Fs-K, capable of entering the host tissue and residing as an endophyte in tomato plants, reduced root pathogen infection and disease development by *F. oxysporum* f. sp. *radicis-lycopersici* and also induced systemic protection against foliar pathogens [32]. SAR, induced biologically and chemically in plants, is associated with an ability of plants to resist pathogen attack by enhanced activation of cellular defense mechanisms [26].

The results indicate that the induced resistance observed in tomato against *Fol* may be a case of SA-dependent SAR. Root feeding of SA against the root pathogen may have induced local resistance in the roots, but leaf feeding of SA clearly demonstrates induction of SAR in the remotely located systemic root tissues. SA feeding does reduce susceptibility of tomato plants to *Fol*, likely due to induction of SAR accompanied by increased activities of the defense enzymes PAL and POD. The results of this investigation show that SA may be used as a potential inducer of SAR against the devastating soil-borne vascular wilt pathogen of tomato.

4. Materials and methods

4.1. Chemicals

Analytical grade chemicals were used in sample preparation and all solvents used for HPLC and LC–MS/MS were of HPLC and LC–MS/MS grades. Deionized water for all procedures was obtained from

a Barnstead/Thermolyne (Iowa, USA) Diamond-Nanopure™ water purification system. Standard salicylic acid was procured from Sigma–Aldrich (New Delhi, India).

4.2. Treatment of tomato plants with SA

Tomato plants (*Solanum lycopersicum* cv. Arka Saurabh) were grown in hydroponics culture according to Spletzer and Enyedi [14] with suitable modifications. After the plants were established in the hydroponics culture, salicylic acid (SA) dissolved in deionized water with 10% methanol was added to the hydroponics medium for root feeding at a final concentration of 200 μM. Spletzer and Enyedi [14] observed that introduction of 200 μM SA in MS medium to the hydroponically grown tomato plants caused no change in leaf turgor or signs of phytotoxicity on the foliage. This is the basis for selection of the SA concentration of 200 μM in the study. Equal volume of deionized water with 10% methanol was added to the hydroponics medium of control plants. Nutrient medium containing SA was exchanged daily for 7 days to ensure a continuous supply of nutrients and required concentration of SA.

The same concentration of SA was sprayed on leaves for leaf feeding on hydroponically grown plants. The spray of SA was repeated every 24 h for 7 days in order to provide adequate absorption of SA by the leaves. Control plants were sprayed with equal volume of deionized water.

4.3. Pathogen inoculation

F. oxysporum f. sp. *lycopersici* strain 1322F was obtained from Indian Type Culture Collection, New Delhi. The pathogen was grown on potato dextrose agar medium in light at 26 °C. The spore suspension used for inoculation was prepared from a 2-week old culture and was applied at a concentration of 5×10^6 spores per 1 mL. Tomato plants treated with 200 μM SA through roots and foliar spray as well as control plants were inoculated with addition of the spore suspension in the hydroponics medium after two days (i.e. 48 h) of last SA application. As control, noninoculated tomato plants were used. Plants were maintained in a growth chamber [33].

4.4. Extraction of SA from the roots samples

To determine if endogenous root levels of SA changed following exogenous root feeding and foliar spray with 200 μM SA, root samples were harvested at 0, 24, 48, 72 and 168 h after last SA application. Roots were obtained from all portions of root system, cut into 5 mm pieces, and combined to ensure heterogeneity of tissue age. Extraction of SA was performed according to a method developed by Verberne et al. [34].

4.5. Determination of SA by HPLC analysis

Detection of SA was done according to Saikia et al. [16] with suitable modifications. SA was detected by HPLC at 275 nm and 310 nm with a Waters Symmetry C_{18} column (3.5 μm, 75 × 4.6 mm; Waters, Symmetry®). A mixture of methanol and 1 mM aqueous trifluoroacetic acid (60:40) was run isocratically with a flow rate of 1 mL min^{-1} for 12 min at room temperature to elute the compound. Twenty microliters of each sample were injected into the column. Identification of SA was achieved by comparing its retention time with that from authentic standard SA. The quantity of SA was computed from the standard curve made with known concentrations of SA and expressed as ng g^{-1} FW.

4.6. Confirmation of identification of SA by LC–MS/MS analysis

Identification of endogenous root SA detected through HPLC was confirmed with LC–MS/MS analysis [35]. The analysis was performed with an ABS 4000Qtrap LC–MS/MS system coupled to Agilent 1200 series HPLC. The transitions used were 195 > 172, 195 > 154 and 195 > 110. Ion source was ESI +ve and ion source temperature was 500 °C. The HPLC conditions: column, Purosphar STAR RP_{18} (55 mm × 2 mm × 3 μm); solvent, acetonitrile:water (0.1% formic acid); flow rate, 300 μL min^{-1}.

4.7. Determination of PAL and POD activities

PAL enzyme extraction from the root samples was carried out at 4 °C. Phenylalanine ammonia lyase was assayed directly in the supernatant after concentration through AmiconR™ Ultra-4 CFU membrane (Millipore, Bedford, USA) by detecting the formation of *t*-cinnamic acid at 280 nm with HPLC [36]. Protein concentration in enzyme extract was measured according to Bradford method.

POD activity was determined from the crude enzyme extract using an assay system consisting of 20 mM guaiacol (0.5 mL), 0.1 mM acetate buffer (pH 5.0) (2.1 mL), 40 mM H_2O_2 (0.2 mL) and the enzyme extract (0.2 mL) with a final volume of 3 mL. Oxidation of guaiacol was measured by the increase in absorbance at 470 nm in a Systronics UV–vis scanning spectrophotometer (Amhedabad, India). One unit of enzyme activity represented the amount of enzyme catalyzing the oxidation of 1 μmol of guaiacol in 1 min [36].

4.8. Disease assessment

Assessment of disease severity was done according to Ishikawa et al. [6] with modifications. Four weeks after challenge of tomato plants by *Fol*, the disease index (on 1–5 scale) on each plant was recorded according to vascular browning and the mean value was calculated as the disease severity. For evaluation of vascular browning, the basal stems were cut and vascular browning was rated on a scale where 1 = no symptoms or vascular browning; 2 = 1–25% vascular browning; 3 = 6–50% vascular browning; 4 = 51–75% vascular browning; 5 = more than 75% vascular browning. Similarly, the disease index as regards to leaf yellowing was recorded on the same scale.

4.9. In vitro antifungal activity assay of SA against Fol

The direct effect of SA was tested on growth of *Fol* reared on potato dextrose agar (PDA) medium. The PDA was amended with SA at 100 μM, 200 μM and 300 μM concentrations. The fungal colony diameters were measured at 9th day after inoculation with a 2 mm diameter plug of *Fol* [14].

4.10. Statistical analysis

Growing, inoculation and sampling of plants were done in three independent experiments with three replicates. For SA quantification and enzyme assays, roots of three plants were considered as 1 sample. Collected plant material was randomly divided into three parts and analysed. For disease assessment, a minimum of six plants were evaluated for each replicate. Statistical analysis was done using Student's *t*-test, with level of significance $P < 0.05$; SD was calculated and its range is shown in the figures.

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