University of Benghazi
Faculty of Education Al marj
Global Libyan Journal

جامعة بنغازي
كلية التربية المرج
المجلة الليبية العالمية

العدد الثاني – مارس 2015

Role of salicylic acid in systemic resistance induced by *Pseudomonas fluorescens and Trichoderma harzianum* against *Alternria solani* in tomato

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Abstract.

Tomato growers are suffering all over the world from massive losses due to root rot diseases, the study aims mainly to study the effect of treatment with many safe fungal and bacterial biological control agents as indicative signal particles to induce host systemic resistance under greenhouse conditions. Some essential parameters and evidences of resistance were determined, i.e. accumulation of salicylic acid (SA).

One week after inoculation tomato seedlings with *A. solani*, previously pretreated with the tested biological inducers, endogenous SA content showed significant increase in all the tested cultivars. Moreover, SA content in resistant cvs were higher, compared with susceptible cvs (1.04-fold over that of susceptible CV).

Key word: Pseudomonas fluorescens – Trichoderma harzianum- Salicylic acid – Alternria solani

الملخص العربى:

يعاني زارعي الطماطم في كل أنحاء العالم من خسائر جسيمة ناجمة عن الاصابة بمرض وعفن الجذور و تهدف الدراسة الى اختبار المعاملة بالعديد من عوامل المكافحة البيولوجية الفطرية والبكترية الآمنة إشارية لحث مقاومة العائل الجهازية في الصوبة وتم اختبار كفاءة هذه العوامل البيولوجية بتقدير بعض أدلة المقاومة كتجمع حمض الساليسيليك .بعد أسبوع من معاملة بادرات الطماطم بالحاثات البيولوجية، زاد المحتوى الداخلى من SA فى جميع الاصناف المختبرة وذلك فى تجربة أ.سولانى.

فقد حققت معاملة التريكودرما هارزيانم أعلى قيمة SA فى تجارب أ.سولانى (الصنف المقاوم تيزر 2.1 ضعف الصنف الحساس كاسل روك).

بعد أسبوع من حقن بادرات الطماطم الممرض أ.سولانى والمعاملة مسبقا بالحاثات البيولوجية المختبرة. أظهر المحتوى الداخلى SA زيادة معنوية فى جميع الأصناف المختبرة.علاوة على ذلك، زادت محتويات SA فى الصنف المقاوم مقارنة بالصنف الحساس, حيث كانت 1.04 ضعف قدر الصنف الحساس.

- **Introduction**

In recent years, strains of *Pseudomonas* and *Trichoderma sp* have been extensively used for plant growth promotion and disease control. Several mechanisms have been suggested for disease control of air borne pathogens by *P. fluorescens* and *Trichoderma sp*, involves production of siderophores, HCN, ammonia, antibiotics, volatile compounds etc. or by competing with pathogens for nutrients or colonization space (Glick 1995). In most of the cases *P. fluorescens* and *Trichoderma sp trigger* a plant-mediated resistance mechanism called induced systemic resistance (ISR; Pieterse *et al*. 1996; Sticher *et al*. 1997; Van Loon *et al*. 1998; Benhamou *et al*. 2000). Rhizobacteria-mediated ISR has been reported for bean, carnation, cucumber, radish, tobacco, tomato and the model plant *Arabidopsis thaliana*, and effective against different types of plant pathogens (Metraux *et al*. 1990; Kwack *et al*. 2002). ISR has many similarities to pathogen-inducible defense called systemic acquired resistance (SAR), which renders uninfected plant parts more resistant towards a broad spectrum of pathogens (Ryals *et al*. 1996; Sticher *et al*. 1997). ISR occurs via a different metabolic pathway from that SAR, which involves salicylic acid (Hoffland *et al*.1996; Pieterse *et al*. 1996; Vindal *et al*. 1998).

Thus, the ISR signaling pathway clearly differs from the one controlling pathogen-induced SAR. The state of SAR is characterized by an early increase in endogenously synthesized salicylic acid (Metraux *et al*. 1990). Salicylic acid (SA) is a phenolic compound that affects a variety of biochemical and molecular events associated with induction of disease resistance. SA has been shown to play an important role in expression of both local resistance controlled by major genes and systemic induced resistance developed after an initial pathogen attack (Hammerschmidt and Smith-Becker 2000). A question is often raised as to whether there are one or several SA signal transduction pathways mediating various defense or several signal related mechanisms. Application of exogenous SA at a concentration of 1 to 5 mM has been long known to induce pathogenesis- related (PR) gene expression and acquired resistance against a variety of microbial pathogens (Ward *et al*. 1991; Meena *et al*. 2001). Low concentrations (10 to 100 μM) of SA have also shown to be sufficient for pathogen-induced defense gene expression, H2O2 accumulation and hypersensitive cell death in plant suspension cultures (Levine *et al*. 1994; Kauss and Jeblick 1996).

Many studies indicated that SA accumulation was associated with plant physiological responses to pathogen infection. Malamy *et al*. (1990) reported that SA level increased as much as 20 fold after TMV infection on resistant cultivar tobacco leaves. Exogenous SA was found to induce PR-protein accumulation in tobacco and this accumulation correlated with increased TMV resistance (Van Loon and Antoniw 1982). Some studies indicated that SA may not be a translocated primary signal for SAR, and SA may only play a regulatory role in the expression of SAR genes (Vernooji *et al*. 1994;

Pieterse *et al*. 1996; Seah *et al*. 1996). They showed that SA may not be involved in all cases of systemic resistance and did not act as exogenous inducers against plant pathogens. In general, SA may play a more important role for SAR than for ISR. However, SA still is an uncertain factor as a signal for SAR or ISR. It is also not clear whether SA is produced in infected plants or by PGPR strains and what role does SA play in resistance to Tomato disease.

• Materials and methods

Plant material, biological control agents and pathogen inoculum

Commercial tomato cultivars, Castle Rock, and Riogrand cvs were obtained from Libyan seed markets.

A. solani was introduced from Mycological Center, Assiut University

Two biological control agents, *Pseudomonas fluorescens* and *Trichoderma harzianum* were obtained from Agricultural research Center, Giza, Egypt.

Tomato seeds of Castle Rock, and Riogrand cultivars were surface sterilized with 2% sodium hypochlorite solution for 2 min., rinsed in sterile distilled water, dried between folds of sterilized filter paper and then sowed in trays containing sterilized peat: sand: clay, 1:1:1 for three weeks. Nursery was irrigated when needed. Seedlings were then transferred to 14 cm diameter pots. Pots were sterilized by sub-immersing in 7% formaldehyde solution for few hours and then left to aerate. The soil was autoclaved and left to aerate. Pots were filled with the mixture of 1:2:1 sand: clay: peatmos and seedlings were planted and placed in the greenhouse at 12:12 hours light: dark cycle, with 24-26 ˚C: 16-18 ˚C days: night temperature and about 65% relative humidity.

For long-term storage *P. fluorescens* strain was maintained at -80 C^{o} in Tryptic Soy Broth (TSB) with the addition with 20% (vol/vol) glycerol. For experimental use, strain was isolated onto Tryptic Soy Agar media (TSA) and was incubated at 28 °C for 24 hours, then transferred to TSB and placed in a shaker at 150 rpm for 24 hrs. The cultures were centrifuged at 6000 rpm for 5 minutes, and suspended in MgSO4 and population was adjusted to 5 x 10^{8} colony forming units (cfu) as measured spectrophotometrically (Zhender *et al.*, 2000).

T. harzianum isolate was cultivated on PDA at 25 C^{o} and maintained at 4 C^{o}.The propgules (colony forming unit, cfu) suspension of *T. harzianum* was prepared in sterile distilled water from 7-days-old-culture on potato dextrose agar (PDA) **(**Rojo *et al.*, 2007). The fungal inoculum was harvested by flooding the culture with sterile distilled water (SDW) (10 ml), and then rubbing the culture surface with a sterile glass rod. The concentration of Trichoderma strain was adjusted to 5 x 10^{7} spores/ml^{-1} by dilution and direct counting using a haemocytometer) **(**Abd El- Khair and El -Mougy, 2003).

The fungal isolate of *A. solani* was kept on PDA slant in deep freezing for maintaining it until used. The purified fungal isolate from PDA slant was renewed on fresh PDA plates incorporated with an antibiotic containing, Benzathine penicillin G 300,000 U, and Terramycin 100 mg, at appropriate concentrations. The plates were incubated for 10 days under 12 hrs fluorescent/ 12 hrs darkness cycle at 25˚C and examined periodically.

salicylic acid (Sigma Chemicals, USA) were prepared in deionized water.

- **Plant growth and disease induction treatment**

Tomato seeds (one gram) were soaked in 10 ml of the bacterial suspension (10^8 cfu /ml) or fungal spore suspension (10^7conidia/ml) in 100 mg of carboxy methyl cellulose in Petri plates. Seeds were air dried for 12 hours then, the suspension was drained off and the seeds were dried overnight in sterile Petri plates (Ramamoorthy *et al.*, 2002).

To support the seed treatment, a root dipping was conducted: roots of tomato transplants were immersed in suspension of each *P.fluorescens* (10^8 cfu/ml) or *T.harzianum* (10^7 conidia/ml) two weeks after seeding for 2 hours (Nawar, 2005).

As a control treatment, water was used instead of bioagent inducers solution in each case. Sets of five pots each, with ten seeds were used for each cultivar tested.

- **Inoculation and preparation of samples**

Tomato plants (45 days-old) were inoculated with spore suspension of isolate of *A.solani* Each treatment includes 5 pots for each cultivar each containing 5 seedlings, whereas two pots left as control. A modified technique of Fritz (2005) was applied through this experiment. Fungal spores of 10 day-old cultures were harvested by gentle brushing to separate the spores from the mycelium surface and then rinsed with a 0.01% Tween 20 solution. The resulting spore suspension was quantified using a haemocytometer to 10^4 spores/ ml^{-1}. Plant leaves were dusted with carborundum crystals and inoculated by spraying the spore suspension until run-off. To ensure good spore germination, the plants were covered with transparent plastic bags for 24 hours to increase the relative humidity. Inoculation of plants with *A. solani* was carried out one week after last treatment with bioagent inducers. Individual leaves from similar positions on each plant were collected. These samples were then used for the preparation of total leaf homogenates to be analysed by HPLC.

- **Effect of SA on mycelial growth.**

A Petri-plate test was carried out to observe the direct effect of SA on the growth of *A.solani*. Actively growing *A.solani* (5 mm disc) was placed at the center of each SA-amended plates containing potato dextrose agar medium (PDA, (pH 5.5). The SA

concentrations in the medium were 0, 50, 100, 200, 500, 1000 and 2000 µg ml^{-1}. The diameter of colony on SA plate was measured 5 day after inoculation from the inoculum disc edge to the growing colony edge. Water was used as a nontreated control.

- **Statistical analysis**

The data were analyzed as (LSD) test ($P \leq 0.05$).

- **Extraction of free SA**

Free SA was extracted from tomato plants according to the method of Malamy and Klessig (1992). One gram of leaf material was frozen in liquid nitrogen, ground in a mortar and homogenized in 3 ml of 90% methanol containing 0.9 g sand. The mortar was washed twice with 2ml of 90% methanol. After centrifugation at 20 000 ×*g* for 15 min, the supernatant was collected and the pellet was again extracted with 4 ml of 90% methanol. After a further centrifugation, the supernatants were combined and evaporated at 40 C° under vacuum and then the residues resuspended in 50 µl of 5% trichloroacetic acid and 1 ml of 100% methanol. The volume of the extract was adjusted with H_2O to 5 ml and centrifuged at 3000 ×*g* for 10 min. The supernatant was applied to a high-performance liquid chromatography.

- **Determination of SA**

Samples (50 µl) were analyzed by HPLC-electrospray ionization using an Agilent 1100 HPLC coupled to an Applied Biosystems Q-TRAP 2000 (Applied Biosystems, California, USA). Chromatographic separation was carried out on a Phenomenex Luna 3 µm C18 (2) 100 mm × 2.0 mm column, at 35°C. The solvent gradient used was 100%A (94.9% H_2O: 5% CH_3CN: 0.1% CHOOH) to 100%B (5% H_2O: 94.9% CH_3CN: 0.1% CHOOH) over 20 min. Solvent B was held at 100% for 5 min then the solvent returned to 100% A for 10 min equilibration prior to the next injection. The solvent flow rate was 200 µl/ min.

To reduce contamination, the first 2 min of the run was directed to waste using the inbuilt Valco valve. Analysis of the compound was based on appropriate Multiple Reaction Monitoring (MRM) of ion pairs for labeled and endogenous SA using the following mass transitions; 2H4 SA 141 > 97, SA 137 > 93, SA-glyc 299 > 93. Optimal conditions were determined using the Quantitative Optimization feature of the Analyst software both by infusing standard into by syringe pump and injecting standard into a 200 µl/min flow of 50% Solvent A/50% Solvent B. The optimized conditions were as follows; Temperature 400°C, Ion source gas 150 psi, Ion source gas 260 psi, Ion spray voltage -4500 V, curtain gas 40 psi, CAD gas setting 2; the DP (-25 V), EP (-9) and CEP (-2) were held constant for all transitions. Collision energies (CE) and dwell times (DT) were specific for each compound/internal standard pair, the parameters used were SA CE-38, DT 50 ms. Data were acquired and analyzed using Analyst 1.4.2 software (Applied Biosystems).

- **SA standard calibration curve**

A standard HPLC calibration solution of salicylic acid, concentrations; 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 µg were prepared by accurate step-by-step dilutions of stock solution 10 µg by weighing 1 g SA and dissolving in 100 ml methanol. To apply standard curve by computer using Microsoft Excel software, plotting a graph with the peak area which resulted after run on HPLC (y-axis) and concentration (x-axis) results in an equation formatted as follows: y= 16751x + 1.2, where solving for x determines the SA concentration of the sample.

- **Results**

Effect of SA on mycelial growth

A significant negative correlation was observed in concentration of salicylic acid and mycelial growth of *A.solani* in a Petri-plate assay. However, at 100 µg ml^{-1} concentration no inhibition was observed and at a concentration of 2000 µg ml–1 mycelial growth was completely arrested

Salicylic acid (SA) content

The aim of this experiment was to study the effect of treatment of tomato seedlings with the selected BCAs with the least antagonistic effect on the endogenous salicylic acid content in both inoculated resistant and susceptible cvs., under greenhouse conditions.

SA extraction

The retention time for the SA standard measured by HPLC was approximately 7.5 minutes (Figure 1). for SA retention peak, no other peaks were obtained, using this extraction procedure. The SA standard curve was linear.

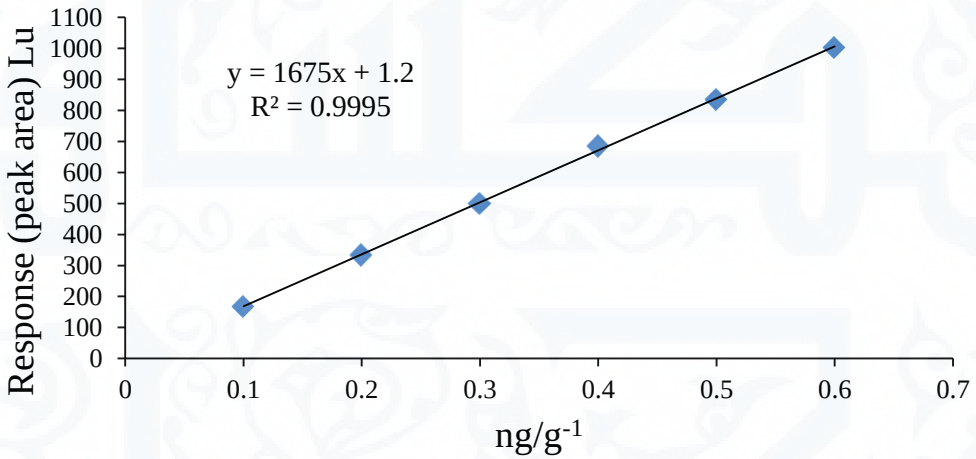
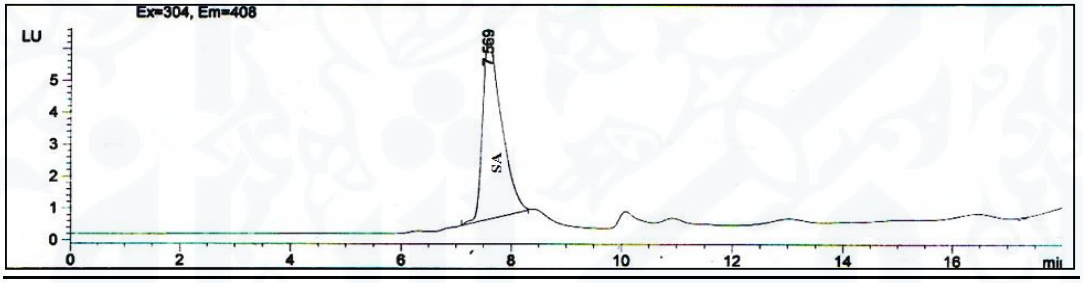


Fig. (1): Salicylic acid (SA) Standard curve

Seven days after treatment

No salicylic acid was detected in untreated control of resistant and susceptible cultivars. Moreover; SA levels in all resistant treatments were, generally, significantly higher than those of susceptible ones.



Ret. Time [min]	Type	Width [min]	Area LU *s	Height [LU]	Area %
7.569	VB	0.3433	136.34943	5.66822	100.000

Fig. 2: Example HPLC chromatogram. Salicylic acid (SA) was extracted from one gram of pretreated tomato leaves of Tezier cv. with *T. harzianum* and inoculated with *A. solani*.

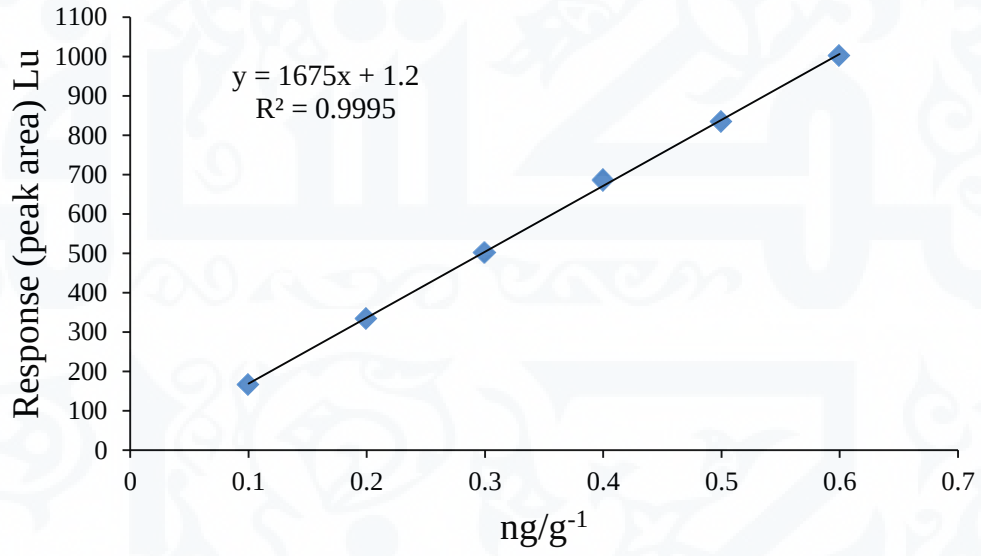


Fig. 3: Salicylic acid (SA) Standard curve

The highest SA level (245 ng/g^{-1} fresh weight) was obtained from Tezier cv. resistant, treated with *T. harzianum*, compared with that of the same treatment in Castle Rock cvs.usceptible (116 ng/g^{-1} fresh weight). In other words SA levels in the resistant cv. was 2.11 times more than in the susceptible tomato cultivar.

A SA level in resistant cv., treated with *P. fluorescens* (230 ng/g-1 fresh weight) was less than that of *T. harzianum* treatment (245 ng/g^{-1} fresh weight). In other words, it constituted 93.9%, of SA content in case of *T. harzianum* treatment.

Unlike the resistant cv. treatments, the highest SA level in susceptible cv. (116 ng/g^{-1} FW) was obtained in seedlings, treated with *T. harzianum*, compared with those of *P. fluorescens* treatment (20 ng/g^{-1} FW).

Seven days after inoculation with *A. solani*

Salicylic acid content in non-inoculated untreated/control of resistant cv. (270 ng/g^{-1} FW) was higher than that of the susceptible cv. control (40 ng/g^{-1} FW). Moreover, SA levels in control treatment was the highest, there were no significant differences with *T. harzianum* treatments (228 ng/g^{-1} FW), while both treatments were significantly higher than of the *P. fluorescens* (28 ng/g^{-1} FW).

The SA level (228 ng/g^{-1} FW) was obtained in inoculated Tezier cv., treated with *T. harzianum*, compared with that of the same treatment in Castle Rock cv. (180 ng/g^{-1} FW), about 1.26 times more than that of the susceptible**.**

A SA level in inoculated resistant cv., treated with *P. fluorescens* (28 ng/g-1 FW) was less than that of *T. harzianum* treatment (228 ng^{-1}/g FW). In other words, they constituted 12.28%, of SA content in *T .harzianum* treatment.

The highest SA level in inoculated susceptible cultivar (180 ng/g^{-1} FW) was obtained in seedlings, treated with *T. harzianum*, compared with these treated with *P. fluorescens* (40 ng/g^{-1} FW).

Table (1): SA concentration (ng/g^{-1} FW) in resistant (Tezier) and susceptible (Castle Rock) cultivars, 7 days after treatment with the tested inducers either *T.harzianum* or *P.fluorescens* and seven days after inoculation with *A.solani,* under greenhouse conditions.

SA content ng^{-1}/g fresh weight(FW)			
Cultivar	**Treatment**	**7days of inducer application**	**7daysa fter pathogen inoculation**
Tezier	Control (H_20)	0.00^a	270^a
	T. harzianum	245^b	228^a
	P. fluorescens	230^c	028^b
Castel Rock	Control	0.00^a	040^b
	T. harzianum	116^d	180^a
	P. fluorescens	020^e	040^b
LSD α=0.05		14.519	104.45

Values followed by the same letter(s) in each column didn't differ significantly according to Scheffe test ($P \leq 0.05$).

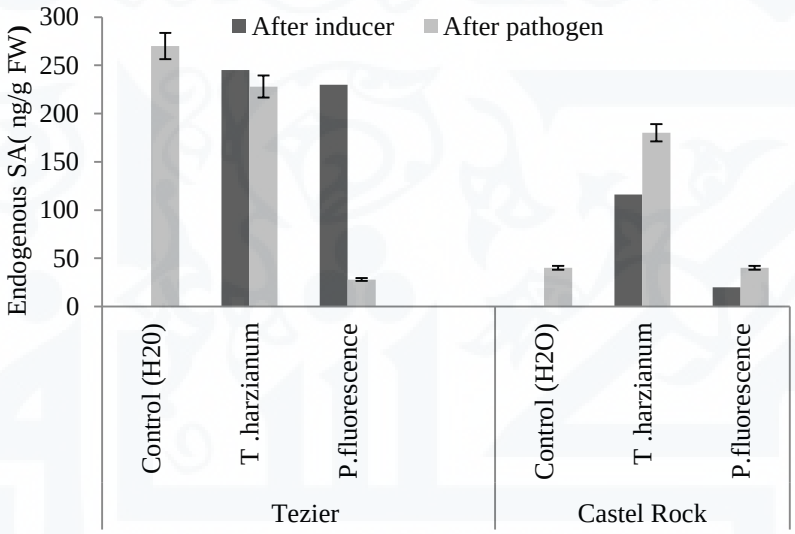


Fig. 4: Endogenous levels of free salicylic acid (SA) in resistant tomato cv. Tezier and susceptible cv. Castel rock after inducer application (dark bars) and after pathogen (*A. solani*) inoculation (shaded bars).

- Leaves were harvested 7 and 14 days respectively, after treatment with H_2O (control) or *T. harzianum* and *P. fluorescens*.
- Data bars are the (means± standard error) of the replicates.

Role of salicylic acid in systemic resistance induced

- **Discussion**

The present data showed that *P. fluorescens* and *T. harzianum* as SAR inducers was based on the spatial separation between bioinducers and fungal pathogen, which exclude the possibility of direct antagonism, leading to the conclusion that systemic resistance was induced (Yang *et al.*, 2009).

Greenhouse experiments showed that treatment with the tested biological inducers *P. fluorescens* and *T.harzianum* significantly increased the accumulation of endogenous SA in both tested tomato diseases in both compatible and incompatible systems. Moreover, Accumulation of SA was more pronounced in resistant Tezier tomato cv.s.This phenomenon was reported by many authors (Audenaert *et al*., 2002; Tripathi *et al*., 2008 and Van den Burg and Takken, 2009). Our findings were also in accordance with those reported by Conrath *et al*. (1995), who concluded that resistant cultivars can accumulate SA more quickly than susceptible ones.

Accumulation of endogenous SA level, throughout the present work, was associated with significant activation of systemic resistance against *A. solani* in tomato plants grown under greenhouse conditions. Such phenomenon was reported in many pathosystems and *P.fluorescens* and *T.harzianum*, were reported as potent inducers of systemic resistance in many plants including; bean, cucumber, tobacco, chickpea and radish (Leeman *et al*., 1996; De Meyer and Hofte, 1997; Chen *et al*., 1998; Notz, 2002; Kubota and Nishi, 2006; Saikia *et al*., 2006 and Caihong and Qian., 2007). Consequently, it was not surprising that these bioinducers can exert a similar effect in tomato; however the magnitude of the increase in SA differed according to the species of the pathogen and the inoculation method used (Saikia *et al.*, 2003).

SA was known to be an important signal molecule and it increases endogenously prior to the activation of SAR in each of the host-pathogen interactions (Malamy *et al*., 1990 and Metraux *et al*., 1990). It was believed that SA has been found to activate through a redox mechanism (Zhang, 2006; Blanco *et al.*, 2009; Hadi and Balali, 2010and Marina *et al*., 2011).

It was found that untreated inoculated tomato plants showed significant increase in endogenous SA levels. These results were in agreement with those reported by Enyedi *et al.* (1992); Malamy *et al.* (1990) and Metraux*et al.* (1990) in many compatible and non-compatible pathosystems. We believe that during early and later stages of infection, pathogen may serve as SAR inducer. The detection limit was approximately 10 ng/g fresh weight of plant tissue, which is sufficient to recognize increases in the SA concentration in the immunized cucumber plants (Kubota and Nishi, 2006). So we conclude that nanogram amounts of SA produced by the *P. fluorescens* and *T. harzianum* activate the SAR pathway. These results were supported by the findings of many authors (Agrawal *et al*., 2002; Kubota and Nishi, 2006 and Martínez-Medina *et al*., 2010). Tameling and Takken (2008) and Truman *et al.* (2010) concluded that plant pathogen interactions are

rapid and dynamic with both host and pathogen constantly wrestling to modify signaling networks and reconfigure metabolism in favor of defense or disease.

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