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Alleviation of Disease Effect on Tomato Plants by Heat Shock and Salicylic Acid Infected with *Alternaria solani*

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ABSTRACT

Eighteen fungal species belonging to 12 genera were isolated from 15 tomato plant samples (*Lycopersicon esculantum* Mill.) collected from Qena Governorate in Upper Egypt. The most prevalent genera were *Aspergillus, Alternaria, Mucor*, and *Cladosporium*. From these genera, *Aspergillus niger, Alternaria solani, Mucor heimales* and *Cladosporium herbarum* were the most common species. *Trichoderma viride* and *Pythium intermedium* were of moderate and remaining species were recovered in low prevalence. Pathogenesity of different isolates of *Alternaria solani* was determined *in vitro* and the highest virulent isolate was used in the subsequent physiological studies. The effect of salicylic acid and heat shock on some physiological responses of tomato plants infected with the virulent isolate *Alternaria solani* was determined. Tomato plants infected with *A. solani* displayed lower values of fresh and dry weights, and pigment contents than the uninfected plants. While, fungal infection enhanced the contents of soluble protein, total free amino acids and proline. Treatment of infected tomato plants with 10^{-6} M salicylic acid enabled plants to tolerate stress due to fungus by increasing the contents of pigments, soluble protein and proline. Therefore, salicylic acid is involved in regulation of resistance against *A. solani*. When tomato plants exposed to heat shock at 40ºC for ½ h, counteracted the adverse effect of fungus on growth parameters which were accompanied by enhanced biosynthesis of pigments, total free amino acids and proline, where the accumulation of free amino acids and proline seems to be on the expense of induced heat shock to soluble proteins.

Key Words: *Alternaria solani*; Heat shock; Mycoflora; Pathogenesis; Proline; Protein; Salicylic acid; Tomato

INTRODUCTION

Mould contamination can occur on a crop during development, harvest, storing, terminal shipment and processing. Many investigations have been reported on mycoflora (El-kady *et al*., 1979; Saber *et al.*, 1994; Abdel-Mallek *et al*., 1995; Perveen & Ghaffar, 1995) and pathogenic fungi on tomato plants (Jones *et al*., 1991; Oladiran & Iwu, 1993; Lawrence *et al*., 1996). In Egypt, Saber *et al.* (1994) identified 22 species contributed to 7 genera from 21 tomato paste samples. They found that *Aspergillus fumigatus, A. flavus, A. niger and Penicillium oxalicum* were predominant on glucose-Czapek's agar medium. While, *Aspergillus niger, A. flavus, A. sydowii* and *Eurotium montevidensis* were the most common species on 10% NaCl glucose-Czapek's agar medium. Perveen & Ghaffar (1995) isolated 37 species of fungi belonging to 20 genera from 24 samples of tomato seeds collected from different parts of Pakistan. The most common fungi were *Fusarium solani, F. moniliforme, Aspergillus flavus, Alternaria alternata* and *Drechslera australiensis* were predominant. The occurrence and distribution of fungal flora on different plant species were also studied in Upper Egypt (Moubasher *et al*., 1972; Abdel-Hafez *et al*., 1996; Mohawed *et al*., 2001; Abdel-Sater & Eraky, 2002).

Being non-motile, tomato plants are often exploited as a source of food and shelter by a wide range of parasite including viruses, bacteria, fungi, nematodes, insects and even other plants. However, they have developed remarkable strategies to adapt to environmental changes by using a range of constitutive or inducible biochemical and molecular mechanisms. They exhibit both long- and short-term defense responses to immediate challenges such a pathogen attacks. Nevertheless, a synergic effect of many stresses represents the primary cause of crop loss. The estimated loss caused by pathogens is typically around 10 to 20% (Boyer, 1982; Scheel, 1998; Nimchuk *et al*., 2001).

Plants have evolved complex, integrated defense mechanisms against disease that include performed physical and chemical barriers, as well as inducible defenses such as the production of anti-microbial compounds, enhanced strengthening of cell walls and the production of various antifungal proteins (Lamb *et al*., 1989; Jackson & Taylor, 1996). These systems form an effective defense against infection, with disease resulting as a rare outcome in the spectrum of plant-microbe interactions. Infectious disease can result when a pathogen is able to overcome the defense processes of a host plant by either actively suppressing or out competing them.

Adverse factors can be either biotic or abiotic factors include bacteria, fungi, insects, or disease-causing organisms. They elicit changes in host genetic expression so that stress-specific compounds are synthesized to enhance host resistance to the foreign organism. Abiotic factors

include temperature, excess water (Ben-Zioni *et al*., 1967; Hsiao, 1970), salinity (Ben-Zioni *et al*., 1967), heavy metals (Jackson *et al*., 1984; Curle & Kapoor, 1988; Gruhn & Miller, 1991), growth regulator (Heikkila *et al*., 1984), ultraviolet irradiation (Chappell & Hahlbrock, 1984), Famine (Curle & Kapoor, 1988), pH (Le John & Braithwaite, 1984). Among the environmental stressors listed above, thermal stress has been most widely studies. Both heat shock and cold shock can induce the synthesis or storage of a group of proteins which increase resistance to thermal to thermal stress (Ketola-Pirie & Atkinson, 1983; Yacoob & Filion, 1987).

The plant hormones salicylic acid (SA), Jasmonic acid (JA), and ethylene (ET) play key roles in the regulation of defense, because plant genotypes that are affected in their response to any these signals are more susceptible to infection by certain virulent pathogens (Hutcheson, 1998; Daugl & Jones, 2001; Lan *et al.,* 2001). Salicylic acid (SA) plays a central role in the signaling pathways involved in systemic acquired resistance (SAR) (Vernooij, 1994; Dong, 1998).

Plant respond to temperature changes through several mechanisms such as synthesis of heat and cold shock protein (Howarth & Skot, 1994; Gimalov *et al.*, 1996; Sabehat *et al*., 1996), and amino acids (Chapin, 1991; Santarius, 1992). According to Delauney and Verma (1993), Arora and Saradhi (1995), Hare *et al.* (1998), Fabro *et al*. (2004), Souza *et al.* (2004) proline accumulation in plant tissues may increase the plant tolerance to several stress, such as biotic or abiotic stress.

During the last 20 years this substance drew the attention of researchers due to its ability to induce systemic acquired resistance (SAR) in plant to different pathogens, which is manifested in the appearance of pathogenesis related proteins (PR). (Pieterse *et al*., 1998), suggesting that the basal resistance pathogen is controlled by action of SA In addition proline accumulation was faster and stronger when stimulated by adding low concentration of salicylic acid to plants (Fabro *et al.,* 2004).

Heat-shock response of microorganisms has been observed by various investigators (Linton *et al*., 1992). They indicated that microorganisms after exposing to a few degree centigrade above their growth temperature might increase their resistance to heat treatment or other stress that were normaly letheal to them however it is reported that a number of factores including growth phase preincubation temperature, pH and heating medium may influence the extent of heat-shook response of micoorganismes (Macky & Derrick, 1990).

The present investigation was aimed to study the occurrence of tomato mycoflora and the effect of heat shock and salicylic acid on biochemical parameters of tomato infected with *Alternaria solani.*

MATERIALS AND METHODS

Tomato mycoflora. Fifteen tomato (*Lycopersicon esculentum* Mill.) plant samples were collected from Qeft, Qus, Naga-Hamady, Deshna and Qena at Qena Governorate, Upper Egypt. These tomato samples were collected at the end of February 2003 from the cultivated fields and brought to the laboratory in clean plastic bags. Tomato leaves from each plant sample were cut into small segments (1 cm) and four segments from each plant sample were cultured on sterile GCA medium in each Petri-dish (Moubasher *et al.,* 1972). Four replicates were used for each plant sample and the dishes were incubated at 24ºC for one week. Then, the colonies of fungi which developed around the samples were examined and identified according to Moubasher (1993), Nelson *et al.* (1983), Lawrence (1989), Zycha (1963), Domsch and Gams (1972). The identified fungi were purified on GCA medium, maintained on slopes of the same medium and stored at 8-12ºC.

Pathogen and infection. *Alternaria solani* (Ell. and Mart.) is the causal agent of early blight in tomato. Nine isolates of *A. solani* were recovered from nine tomato samples (**Table I**). The pathogenicity of these isolates were examined in tomato cv. Peto-86 (*Lycopersion esculentum* Mill.) as described by Thanutong *et al.* (1983) and Khan (2002). The isolates were cultured on Glucose Czapek,s agar medium (GCA) and incubated in the dark at 24ºC. For inoculation and disease assessment, tomato seeds were surface-sterilized by soaking in 5% sodium hypochlorite solution for 2 minutes and then washed thoroughly in a sterile water. The sterilized seeds were cultured in aseptic closed 125 ml bottles (for high humidity), each bottle contains 25 ml of ¼ MS-medium (Murashige & Skoog, 1962) and incubated under semi-controlled conditions in the green house for 4 weeks. Four week old plants were sprayed uniformly with spore suspension (10^6 ml^{-1}) from each isolate and maintained at 25ºC under continuous light for one week. After two weeks of inoculation, percentage of diseased plants was determined for each isolate of *A. solani*. The pathogenic isolates of *A. solani* were re-isolated from the infected tissues and kept in pure cultures.

Physiological studies. The effect of salicylic acid and heat shock on some physiological characters of tomato plants infected with the virulent isolate (No.5) of *A. solani* was investigated. Tomato seeds of cultivar Peto-86 were surface sterilized with sodium hypochlorite solution (5 %) and grown aseptically in 125 ml bottles containing 25 ml of ¼ MS medium (Murashige & Skoog, 1962) free of growth hormones. Fifteen sterile seeds were grown in each bottle and maintained in controlled environment cabinet at 25 ± 1^0C with 12 hr light period.

Treatments. After four weeks of incubation, 30 bottles with healthy grown plants were inoculated with the pathogenic isolate of *A. solani.* Additional 10 bottles remain without inoculation (control). The cultures were incubated for 10 days at 25^0C. Afterwards, inoculated bottles were divided

Table I. Fungal species were isolated from 15 samples of tomato plants collected from different fields in Qena Governorate, Upper Egypt

Fungal Species	Qeft			Qus			Qena			Naga- Hamady			Deshna			TC	TC %	OR
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3			
Aspergillus	2	9	2	16	10	1	5	5	2	8	5	3	7	2	2	82	19.9	H
A. ustus(Bainier)Thom et Church										3						3	0.73	L
A.flavus (Link)										3			2			5	1.21	L
A.niger (van Tieghem)	2	9	2	14	3	1	5	5	2	2	5	3	5	2	2	62	15.0	H
A.terrus (Thom)				2												2	.48	L
Alternaria solani (Ellis et Martin)					7			14	4	6	14	6	16	2	2	81	19.7	H
Cladosporium herbarum (Link)Fries		3	3				6	4		6			4	7	5	37	8.98	H
Curvularia lunata (Wakker) Boedijn						20										20	4.85	L
Helminthosporium sativum (Pammel *et al*.)					3	14							18			35	8.49	L
Mucor hiemalis (Wehmer)	4	6	10	4	4		4	3	4		4					43	10.4	H
Paecilomyces variotii (Bainier)										3		4		2		9	2.18	L
Penicillium rubrum (Stoll)				8			12		3							33	8.0	L
Penicillium notatum (Westling)				2				1	2							5	1.21	L
Penicllium citrinum (Thom)	5		3					4								12	2.91	L
Pythium intermedium (de Bary)			2			1				3			3			9	2.18	M
Pyth. Aphanidermatum (Edson)Fitzpatrick)						3							3			6	1.45	L
Rhizopus nigricans (Ehrenberg)									3	19			4			26	6.31	L
Trichoderma viride (Persoon;fries)			3							6		4	3			16	3.88	M
Torula herbarum (Persoon)Link					3					2			3			8	1.94	L

High occurrence: 8 - 15
Moderate occurrence: 4-7
Low occurrence: 1-3.

into 3 groups. The first group exposed to 40^0C for 1/2 hr while each bottle in the second group was supplied with 1.0 ml of sterile 10^{-6} M salicylic acid and third group of inoculated bottles was without additional treatments. All bottles were incubated at 25^0C for one more weak.

At the end of experiment, fresh and dry weights as well metabolic changes including the photosynthetic pigments (chlorophyll a, b and carotenoids; Metzner *et al*., 1965), free proline (Bates *et al*., 1973), soluble proteins and total free amino acid were determined. Soluble proteins were determined according to the Bradford dye binding method (Bradford, 1976) using bovine serum albumin as a standard, and total free amino acids were analyzed according to Moore and Stein (1948) using L-Lysine as a standard.

RESULTS AND DISCUSSION

Mycoflora of tomato. Eighteen fungal species belonging to 12 genera were isolated from fifteen tomato plant samples collected from Qena Governorate. The most prevalent genera were *Aspergillus*, *Alternaria*, *Mucor*, and *Cladosporium.* In Egypt, Saber *et al.* (1994) identified 22 species contributed to 7 genera from 21 tomato paste samples. However, Perveen and Ghaffar (1995) isolated 37 species of fungi belonging to 20 genera from 24 samples of tomato seeds collected from different parts of Pakistan.

Aspergillus species was the most common genus and occurred in all tested samples constituting 19.9% of total fungal counts. It was represented by four species of which *Aspergillus niger* was the most prevalent (12 samples, 15% of the total counts). The other species were of low occurrence and namely, *A. ustus* (2 samples, 0.73% of the total counts), *A. terrus* (2 samples, 0.48% of total counts) and *A. flavus* (3samples, 1.21% of the total counts). Likewise, El-kady *et al*. (1979) isolated *Aspergillus flavus*, *A. flavus var. columnaris* and *A. niger* from tomato paste. Similarly, Saber *et al.* (1994) isolated 9 species of *Aspergillus* from tomato paste samples including *A. flavus*, *A. niger* and *A. terrus*. Abdel-Mallek *et al.* (1995) isolated *Aspergillus niger* from 84.6% of the tested healthy tomato fruits. However, Perveen and Ghaffar (1995) found that *Aspergillus flavus* was the most common species of *Aspergillus* in tomato seeds.

Alternaria solani come after *Aspergillus* and represented by high occurrence (9 samples, 19.7% of the total counts). *Mucor heimales* was the third genus and represented by high occurrence (9 samples, 10.4% of the total counts). *Cladosporium herbarum* was also represented by high occurrence which appeared in 9 samples constituting 8.98% of the total counts of fungi.

Species from the genus *Penicillium* were isolated from 6 samples constituting 12.14% of the total counts. It was represented by three species namely*; Penicillium citrinum* (3 samples, 2.91% of the total counts), *P. rubrum* (3 samples, 8% of the total counts) and *P. notatum* (3

Table II. Pathogenicity test of nine isolates of *A. solani* on tomato cv. Peto-86

	Isolates of A. solani								
	1	2	3	4	5	6	7	8	9
No. of treated plants	22	20	18	18	22	20	20	24	25
No. of diseased plants	0	1	0	0	4	0	2	0	0
% of diseased plants	0.0	5.0	0.0	0.0	18.2	0.0	10.0	0.0	0.0

samples,1.21% of the total counts). *Pythium* was of moderate occurrence which isolated from 4 samples representing 3.64% of the total fungal counts. It was represented by two species namely; *Pythium intermedium* (4 samples, 2.18% of the total counts) and *P. aphanidermatum* (2 samples, 1.45% of the total counts). *Trichoderma* was also of moderate occurrence (4 samples, 3.88% of the total counts) and represented by one species, *Trichoderma viride* only. All of these species were isolated previously, but with variable densities and frequencies, from tomato (El-kady *et al.*, 1979; Saber *et al.*, 1994; Perveen & Ghaffar, 1995) and other plant species (Abdel-Hafez *et al*., 1996; Mohawed *et al*., 2001; Abdel-Sater & Eraky, 2002). Saber *et al.* (1994) isolated 4 species of *Penicillium* and one species of *Trichoderma* from tomato paste samples including *P. citrinum* and *T. viridi*.

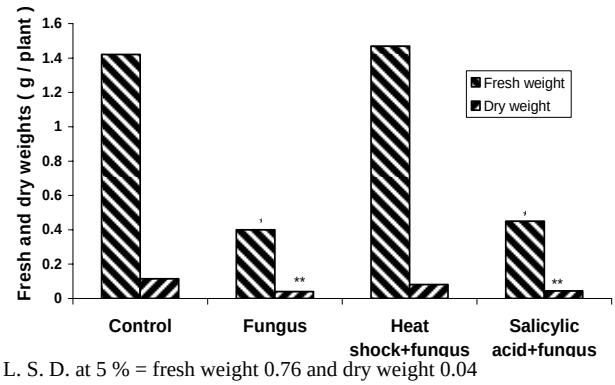
The remaining genera and species of fungi were of low occurrence; *Helminthosporium sativum* (3 samples, 8.49% total counts), Paecilomyces *variotii* (3; 2.18%), *Rhizopus nigricans* (3; 6.31%), *Torula herbarum* (3; 1.94%) and *Curvularia lunata* (1; 4.85%). These species were isolated previously from different plant species, but with variable frequencies (Abdel-Hafez *et al*., 1996; Mohawed *et al*., 2001; Abdel-Sater & Eraky, 2002).

Pathogenicity of *Alternaria solani* isolates. In the present investigation, 9 isolates of *A. solani* were recovered from nine tomato samples. The pathogenicity of these isolates as causal agents to early blight in tomato cv. Peto-86 was tested and summarized in (Table II). The results showed that the isolate No.5 was the most highly virulent followed by No.7 and No.2. Meanwhile, plants inoculated with the other isolates didn't show any symptoms of early blight disease which reflect that these isolates were non-pathogenic. Similarly, Khan (2002) studied the resistance of two tomato species to five isolates of *A. solani.* He found that the five isolates of *A. solani* were non-pathogenic against the tested tomato species.

Physiological studies. Tomato seedlings infected with *A. solani* (isolate No.5) showed significant decreased in their fresh and dry weights than the controls treated with or without salicylic acid or heat shock (Fig. 1), the infected seedlings under heat shock displayed an increase in the growth parameters as compared with their respective control. Infected tomato seedlings also showed highly significant decrease in the contents of chl. a, chl. b, cartenoides and consequently total pigments as compared to the control (Fig. 2). While, this decrease was not pronounced when infected tomato plants exposed to the heat shock or in case of adding salicylic acid to sterile nutrition medium.

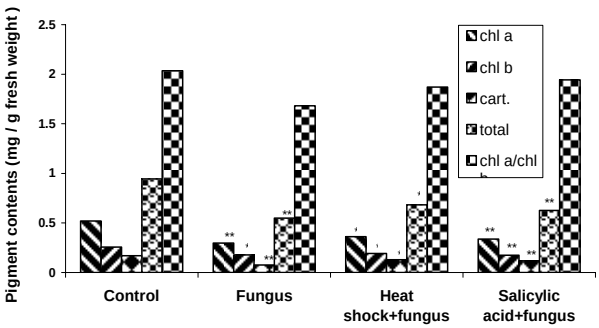
The observed pronounced decrease in growth and pigments, at the infected tomato seedlings lends support to the results obtained in other reports (Boyer, 1982; Fricke & Peters, 2002). De La Rosa-Ibarca and Maiti (1995) suggested that the tender decrease of chlorophyll content might be due to the synthesis of nitrogen compounds. This

Fig. 1. Effect of fungal infection, heat shock (40 ^{0}C for ½ hr.) and salicylic acid (10^{-6} M.) on fresh and dry weights (g/plant) of tomato plants



L. S. D. at 5 % = fresh weight 0.76 and dry weight 0.04
L. S. D. at 1 % = fresh weight 1.16 and dry weight 0.06
* significant differences
** highly significant differences as compared with reference control.

Fig. 2. Effect of fungal infection, heat shock (40^{0}C for ½ hr.) and salicylic acid (10^{-6} M.) on pigment contents (mg/g fresh weight) of tomato plants



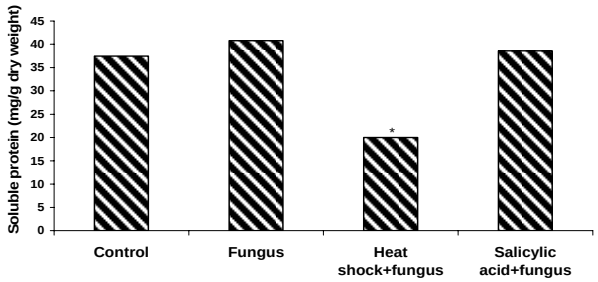
L. S. D. at 5 % = chl a 0.09, chl b 0.05, cart. 0.03, total 0.16 and chl a / chl b 1.
L. S. D. at 1 % = chl a 0.16, chl b 0.08, cart. 0.05, total 0.27 and chl a / chl b 1.66
* significant differences
** highly significant differences as compared with reference control

suggestion is in agreement with the results obtained here, which showed that the decrease in the content of pigments was accompanied by increase of protein, total free amino acids and proline content.

In an attempt by heat shock especially and salicylic acid reduced the fungus stress- induced loss in chl a, chl b and carotenoid contents and promoting the growth probably by increasing the rate of photosynthetic electron transport above the control level and increased the photochemical activity of leaves (Tari *et al.*, 2002). Moreover, salicylic acid increased H_2O_2 derived from the reactive oxygen species produced during abiotic stress (Yalpani *et al*., 1994).

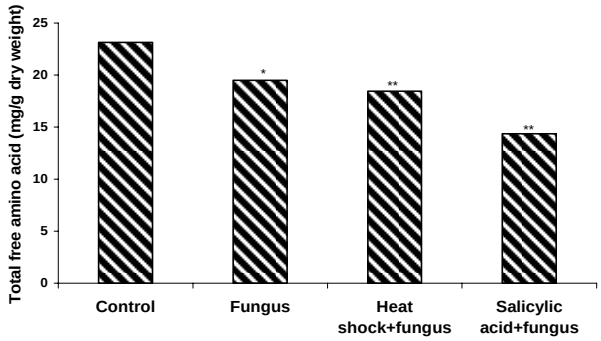
The accumulation of proline seemed faster and stronger under fungal stress (Fig. 5). Rhodes *et al.* (1986), Delauney and Verma (1993) found that proline

Fig. 3. Effect of fungal infection, heat shock (40 ^{0}C for ½ hr.) and salicylic acid (10^{-6} M) on soluble protein (mg/g dry weight) of tomato plants



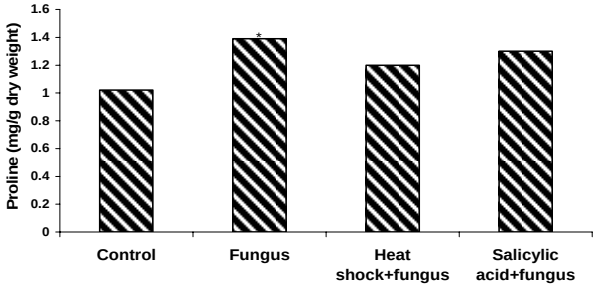
L. S. D. at 5 % = 13.18 and at 1 % = 19.95 .
* significant differences
** highly significant differences as compared with reference control.

Fig. 4. Effect of fungal infection, heat shock (40 ^{0}C for ½ hr.) and salicylic acid (10^{-6} M) on the total free amino acid (mg/g dry weight) of tomato plants



L. S. D. at 5 % = 3.11 and at 1 % = 4.7
* significant differences
** highly significant differences as compared with reference control.

Fig. 5. Effect of fungal infection, heat shock (40 ^{0}C for ½ hr.) and salicylic acid (10^{-6} M) on the proline (mg/g dry weight) of tomato plants



L. S. D. at 5 % = 0.32, and at 1%=0.48.
* significant differences.
** highly significant differences as compared with reference control..

accumulation is thought to function as a compatible osmolyte that stabilizes membranes and subcellular components, including the mitochondrial electron transport complex II (Humilton & Heckathor, 2001). In addition, proline is proposed to scavenge free radicals (Smirnoff & Cumbes, 1989; Saradhi *et al*., 1995; Siripornadulsil *et al.*, 2002) and to ameliorate shifts in redox potential by replenishment of the NADP+supply (Delauney & Verma, 1993; Hare & Gress, 1997). Cross-talk, between aboitic and biotic defense programs have been suggested (Genoud & Metraux, 1999; Singh *et al*., 2002). In addition, proline accumulation seems to be greater when stimulated by applying low concentration of salicylic acid on tomato plants. This conclusion is in agreement with other studies, which documented the importance of salicylic acid in pathogen-induced disease resistance and hypersensitive cell death (Gaffiney *et al*., 1993; Delaney *et al.*, 1994; Avarez, 2000; Fabro *et al*., 2004). Through the potentiation of oxidative burst, salicylic acid can control both biotic and abiotic defense programs (Shirasu *et al*., 1997; Borsani *et al*., 2001).

The accumulation of proline seems to be at the expense of other free amino acid (Fig. 4). This conclusion is in agreement with other studies (Abdel-Samed, 1991; Hamada & Khulaff, 1995), who found that the accumulation of amino acids was nearly opposite to that of proline.

The increase of soluble protein under the effect of fungus stress was the similar by adding salicylic acid, as was previously recorded (Fig. 5). Van Loon (1997) found that, the protein related pathogen form a set of pathogen-induced proteins that may be considered as stress proteins. When salicylic acid added to sterile medium nutrition inoculated by fungus, the increase soluble protein contents thus increase agreement by Van Loon and Antoniw (1982), Van Loon (1997) they observed that exogenous by applied salicylic acids induces both acquired resistance and protein related resistance in e. g. tobacc, tomato and Arabidopsis and when salicylic acid was watered on the soil, aquired resistance was apparent in upper leaves indicating that salicylic was absorbed by the roots and transported throught the plant when the tomato plants exposed to heat shock, after infected by fungus, the soluble protein content changed in comparison with control (Fig. 5). This may be due to a promotion in the conversion of other amino acids and proline seems to be on the expense of the soluble proteins. This conclusion is in agreement with other studies (Howarth & skot, 1994; Gimalor *et al*., 1996; Sabehat *et al*., 1996) indicating that plant responded to temperature changes through several mechanisms such as synthesis of heat and cold shock proteins (Chapin, 1991) and free proline accumulation (Hare *et al*., 1998) in plant tissues may increase the plant tolerance to several stresses.

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