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Quantitative changes in phytochemicals in tomato plant due to application of resistance inducing chemicals and their role in inhibition of early blight pathogen *Alternaria alternata*

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Abstract: Phytochemicals *viz*. soluble protein, reducing sugar and phenols were quantified from tomato (*Solanum lycopersicon*) leaves after application of resistance inducing chemicals *viz*. salicylic acid, β-aminobutyric acid, chitosan and 2,6- dichloroisonicotinic acid as 8 hr seed dip treatment or 2 hr seedling dip treatment or both treatment to study their effect on induction of resistance and inhibition of growth of pathogen. Soluble proteins and phenols were found maximum due to seed+seedling treatment of salicylic acid @ 1.5 mM concentration with 76.90 per cent and 102.68 per cent increase over control whereas reducing sugar was maximum for seed+seedling treatment of β-aminobutyric acid @ 15.0 mM concentration with 61.38 per cent increase over control. The increased level of protein quantity had no effect on inhibition of *Alternaria alternata* growth, whereas the increased quantity of sugar inhibited the average growth of *Alternaria* up to 19.39 per cent. Among phenolic compounds catechol and the cinnamic acid (formed in shikimic acid pathway of phenol biosynthesis) was inhibitory to the *A. alternata* whereas tannic acid had some effect on inhibition of *Alternaria* growth (13.84 % fungal growth inhibition). The increased level of sugar+phenol tested against the pathogen completely inhibited the growth of *Alternaria* fungus. Thus, the increased level of reducing sugar and phenol in tomato leaves due to the application of resistance inducing chemicals seems to be inhibitory to the pathogens multiplication and pathogenesis.

Keywords: *Alternaria alternata*, Inhibition, Phytochemicals, Resistance inducing chemicals

INTRODUCTION

Phytochemicals particularly protein, sugars and phenols present in the plant are known to play a role in conferring resistance to the plant against the disease (Bennett and Wallsgrove, 1994; Reddy *et al.*, 1999; Panina *et al.*, 2007; Rodaki *et al.* 2009; Ashry and Mohammad, 2012). These phytochemicals in the plant system are present in a specific quantity to regulate the biochemical and metabolic process in the plant (Kefeli and Kutacek, 1977; Rosa *et al.*, 2009). Any stress biotic or abiotic, caused to the plant increases the quantities of these phytochemical (Akinwunmi *et al.*, 2001; Samia and Khallal, 2007) to encounter the effect of stress on plant pathogen and some of the chemicals are known to increase these phytochemicals to restrict the pathogen, its growth and disease in the plant (Flors *et al.*, 2004; Maddox *et al.*, 2010; Wu *et al.*, 2010; Ashry and Mohammad, 2012; Moghaddam and Ende, 2012). Some of the chemicals are known as resistance inducers (RI) *viz*. salicylic acid (Spletzer and Enyedi, 1999; Ju *et al.*, 2002; Cheng-bo and Hua-zhi, 2005; Esmail-zadeh *et al.*, 2008; Hadi and Baladi. 2010), β-amino butyric acid (Jakab *et al.*, 2001; Si-Ammour *et al.*, 2003; Arici and Dehne, 2007; Polyakovskii *et al.*, 2008), chitosan (Pospiezny *et al.*, 1991; Lafontaine and Benhamou, 1996; Muzzarelli *et al.*, 2001) and 2,6 dichloroisonicotinic acid (Friedrich *et al.*, 1996). The present investigation was carried out to study the effect of RI on quantitative changes in phytochemicals of tomato plant *viz*. protein, sugars and phenols content and the role in inhibition of *Alternaria alternata*.

MATERIALS AND METHODS

The biochemical constituents *viz*. protein, reducing sugar and phenol in the tomato plant were studied from the tomato plants raised with RI treated seed (8 hr seed dip method), RI treated seedlings (2 hr seedling root dip method) and both RI treated seed+seedling. The tomato plant at 30 days after RI treatment was used for quantification of these chemical constituents as the resistance induced by RIC persisted up to 50 days period of RIC treatment.

Quantification of biochemical constituents from RI treated tomato plant

Soluble protein: Soluble protein content was quantified by the method of Lowry *et al.* (1951). 0.25 g tomato leaf sample of each treatment was macerated separately in a mortar and pestle in 10 ml of water and centrifuged at 10000 x g for 20 min. and the super-

natant obtained was used for protein quantification. One ml of test solution after dilution was mixed with 50 ml of alkaline solution and kept for 10 min at room temperature. Folin-Ciocalteau reagent (0.5 ml) was rapidly added with immediate mixing and the colour intensity was measured after 30 min at 660 nm in spectrophotometer against blank. The protein content was calculated from the standard curve prepared by using various concentrations of bovine serum albumin (BSA).

Reducing sugar: Reducing sugar content was quantified by Nelson Somogyi's method (Somogyi, 1952). 0.25 g tomato leaf samples was macerated in mortar and pestle in 10 ml of 80 % alcohol and centrifuged at 10000 x g for 15 min. The supernatant obtained was used as an enzyme extract for estimation of reducing sugar. One ml of enzyme extract was pipetted in a test tube and 1 ml of alkaline copper tartarate reagent was added to it. The content was mixed and heated for 10 min in boiling water bath. After cooling, 1 ml of arsenomolybdate reagent was added and the contents were diluted to 8 ml by adding 5 ml distilled water. The intensity of the colour was read at 520 nm in spectrophotometer against the blank. The reducing suagr content was calculated from the standard curve against concentration of D-glucose solution.

Total phenol: Total polyphenol content was quantified by using Folin-Denis reagent as described by Swain and Hills (1959). 0.25 g tomato leaf sample of each treatment was separately macerated in a mortar and pestle in 10 ml of water and phenols were extracted by boiling in hot water bath. The contents were centrifuged at 10,000 x g for 15 min. The extraction was repeated twice and the supernatant was diluted to 10 ml. 1 ml of extract was mixed with 7 ml of water and 0.25 ml Folin-Denis reagent and kept for 10 min. After 10 min 1 ml of the alkaline reagent was added in each tube and the content was mixed thoroughly. After 20 min, the content was made to 10 ml and the extinction was measured at 650 nm on spectrophotometer. The concentration of total phenolics was calculated from a standard curve and expressed as mg 100 g^{-1} on a fresh weight basis.

***In vitro* testing of effect of increased level of biochemical constituents on growth of *A. alternate*:** The quantities of sugar, protein and phenols present in normal tomato leaves as well as in the RI treated leaves were tested to see their effect on the growth of *A. alternata* under *in vitro* test.

In a sterilized luke warm simple agar medium sugar (glucose), protein (serum albumin), and phenols (catechol, cinnamic acid, tannic acid) were added separately and combination equal to the quantities observed in normal tomato leaves and in RI treated leaves. The content in flasks were shaken thoroughly and poured in Petri plates (20 ml/ plate). Three plates for each treatment were maintained. After solidification of the medium, each plate was inoculated with eight days old 5 mm *Alternaria* fungal disc. The plates were incubated at room temperature for five days. The colony diameter of the *A. alternata* pathogens on the medium was recorded.

RESULTS AND DISCUSSION

The changes in sugar, protein and phenol quantities in tomato leaves due to different resistance inducing chemicals were studied. The results (Table 1) indicated that the increase in protein (mg/g of fresh leaf sample) in tomato leaves due to different resistance inducing chemical's seed treatment was in the range of 2.49-3.28 mg. The maximum increase (3.288 mg/g fresh leaf sample) in protein content was observed with β–aminobutyric acid @ 15.0 mM concentration. The increase in protein content due to salicylic acid @ 1.0 mM and 1.5 mM concentration was 2.986 and 3.043 mg, respectively over control. The increase in protein content due to β–aminobutyric acid @ 10.0 and 15.0 mM concentration was 2.497 and 3.288 mg respectively. The chitosan and 2,6-dichloroisonicotinic acid increased protein content by 2.554 and 2.799 mg respectively and were statistically significant over control. Similarly seedling treatment with resistance inducing chemicals also increased the protein content in the leaves which was statistically significant over control. Higher increase (4.321 mg) in protein content was observed with salicylic acid @ 1.5 mM concentration followed by β–aminobutyric acid @ 15.0 mM concentration. The maximum increase in protein content was observed to be 4.321 mg. Similar results were obtained when seed + seedling treatment were done with resistance inducing chemicals. The maximum increase was observed for salicylic acid @ 1.5 mM concentration which was 76.90 per cent more over control. The increase in protein was statistically significant for seed, seedling and seed+seedling treatment with resistance inducing chemicals over control.

The increase in reducing sugar (mg/g fresh leaf sample) in the leaves of tomato due to different RI seed treatment was in the range of 1.016-2.192 mg (Table 2) and were statistically significant over control. The maximum increase (2.192 mg) in reducing sugar content was observed with β–aminobutyric acid @ 15.0 mM concentration. The increase in reducing sugar content due to salicylic acid @ 1.0 mM and 1.5 mM concentration was 1.016 and 1.829 mg, respectively over control. The increase in reducing sugar due to β–aminobutyric acid @ 10.0 and 15.0 mM concentration was 1.654 and 2.192 mg respectively. The chitosan and 2,6-dichloroisonicotinic acid increased reducing sugar content by 1.840 and 1.360 mg/g, respectively. Similarly seedling treatment with resistance inducing chemicals also increased the reducing sugar in tomato leaves which were statistically significant over control. Higher increase in reducing sugar content was observed with β–aminobutyric acid @ 1.5 mM concentration followed by chitosan @ 15.0 mM concentra-

Table 1. Protein profile in tomato leaves due to different resistance inducers.

S. N.	Treatment	Conc. (mM)	Protein content in tomato leaves (mg of soluble protein/g of sample)					
			Seed treatment	% increase in protein content	Seedling treatment	% increase in protein content	Seed + seedling treatment	% increase in protein content
1.	Salicylic acid	1.0	8.697 (2.986)	52.29	8.956 (3.226)	56.30	10.079 (4.377)	76.76
2.	Salicylic acid	1.5	8.754 (3.043)	53.28	10.051 (4.321)	75.41	10.087 (4.385)	76.90
3.	β-amino butyric acid	10.0	8.208 (2.497)	43.72	8.620 (2.890)	50.44	9.127 (3.425)	60.07
4.	β-amino butyric acid	15.0	8.999 (3.288)	57.57	9.020 (3.290)	57.42	9.475 (3.773)	66.17
5.	Chitosan	15.0	8.265 (2.554)	44.72	8.466 (2.736)	47.75	9.148 (3.446)	60.43
6.	2,6-dichloroisonicotinic acid	10.0	8.510 (2.799)	49.01	-	-	-	-
7.	Control (Non treated)	-	5.711	-	5.730	-	5.702	-
	SE(m) +_		0.090		0.075		0.066	
	CD (P=0.01)		0.269		0.228		0.200	

Figures in parenthesis indicates increase in protein content (mg) over control

Table 2. Reducing sugar profile in tomato leaves due to different resistance inducers.

S. N.	Treatment	Conc. (mM)	Reducing sugar content in tomato leaves (mg/g of sample) due to					
			Seed treatment	Per cent increase in sugar content over control	Seedling treatment	Per cent increase in sugar content over control	Seed + seedling treatment	Per cent increase in sugar content over control
1.	Salicylic acid	1.0	3.218 (1.016)	46.14	3.617 (1.404)	63.44	3.867 (1.666)	75.69
2.	Salicylic acid	1.5	4.031 (1.829)	83.06	4.140 (1.927)	87.08	4.237 (2.036)	92.50
3.	β-amino butyric acid	10.0	3.856 (1.654)	75.11	3.890 (1.677)	75.78	4.101 (1.900)	86.32
4.	β-amino butyric acid	15.0	4.394 (2.192)	99.55	4.449 (2.236)	101.04	4.461 (2.260)	102.68
5.	Chitosan	15.0	4.042 (1.840)	83.56	4.330 (2.128)	95.66	4.336 (2.135)	97.00
6.	2,6-dichloroisonicotinic acid	10.0	3.562 (1.360)	61.76	-	-	-	-
7.	Control (Non treated)	-	2.202		2.213		2.201	
	SE(m) +_		0.038		0.045		0.033	
	CD (P=0.01)		0.110		0.13		0.101	

Figures in parenthesis indicates increase in reducing sugar content (mg) over control

tion. The Maximum increase in reducing sugar content was observed to be 2.236 mg. Similar results were obtained when seed + seedling treatment were done with resistance inducing chemicals. The maximum increase was observed for β–aminobutyric acid @ 15 mM concentration which was 102.68 per cent more over control. The increase in mg reducing sugar due to various RI treatment was statistically significant for seed, seedling and seed+seedling treatment over control.

The increase in phenol content (Table 3) in the leaves of tomato due to different RI treatment was in the range of 0.038-0.095 mg due to seed treatment. The maximum increase (0.095 mg) in phenol content was observed with salicylic acid @ 1.0 mM concentration. The increase in phenol content due to salicylic acid @ 1.0 mM and 1.5 mM concentration was 0.095 and 0.080 mg respectively over control. The increase in phenol content due to β–aminobutyric acid @ 10.0 and 15.0 mM concentration was 0.051 and 0.057 mg respectively. The chitosan and 2,6-dichloroisonicotinic acid increased the phenol content by 0.044 and 0.038 mg respectively. The increases in phenol content due to seed treatment with resistance inducing chemicals were statistically significant over control. Similarly tomato seedling treatment with resistance inducing chemicals also increased phenol content in the leaves which were statistically significant over the control. Higher increase (0.114 mg) in phenol content was observed with salicylic acid @ 1.5 mM concentration followed by salicylic acid @ 1.0 mM concentration. Similar results were obtained when tomato seed +

Table 3. Phenol profile in tomato leaves due to different resistance inducers.

S. N.	Treatment	Conc. (mM)	Phenol content in tomato leaves (mg/g sample) due to Seed treatment	Per cent increase in phenol content over control	Seedling treatment	Per cent increase in phenol content over control	Seed + seedling treatment	Per cent increase in phenol content over control
1.	Salicylic acid	1.0	0.334 (0.095)	39.75	0.321 (0.088)	37.77	0.353 (0.107)	43.50
2.	Salicylic acid	1.5	0.319 (0.080)	33.47	0.347 (0.114)	48.93	0.397 (0.151)	61.38
3.	β-amino butyric acid	10.0	0.290 (0.051)	21.34	0.277 (0.044)	18.88	0.321 (0.075)	30.49
4.	β-amino butyric acid	15.0	0.296 (0.057)	23.85	0.303 (0.070)	30.04	0.342 (0.096)	39.02
5.	Chitosan	15.0	0.283 (0.044)	18.41	0.290 (0.057)	24.46	0.303 (0.057)	23.17
6.	2,6-dichloroisonicotinic acid	10.0	0.277 (0.038)	15.90	-	-	-	-
7.	Control (Non treated)	-	0.239	-	0.233	-	0.246	-
	SE(m) +_		0.006		0.007		0.008	
	CD (P=0.01)		0.017		0.019		0.023	

Figures in parenthesis indicate increase in phenol content (mg) over control

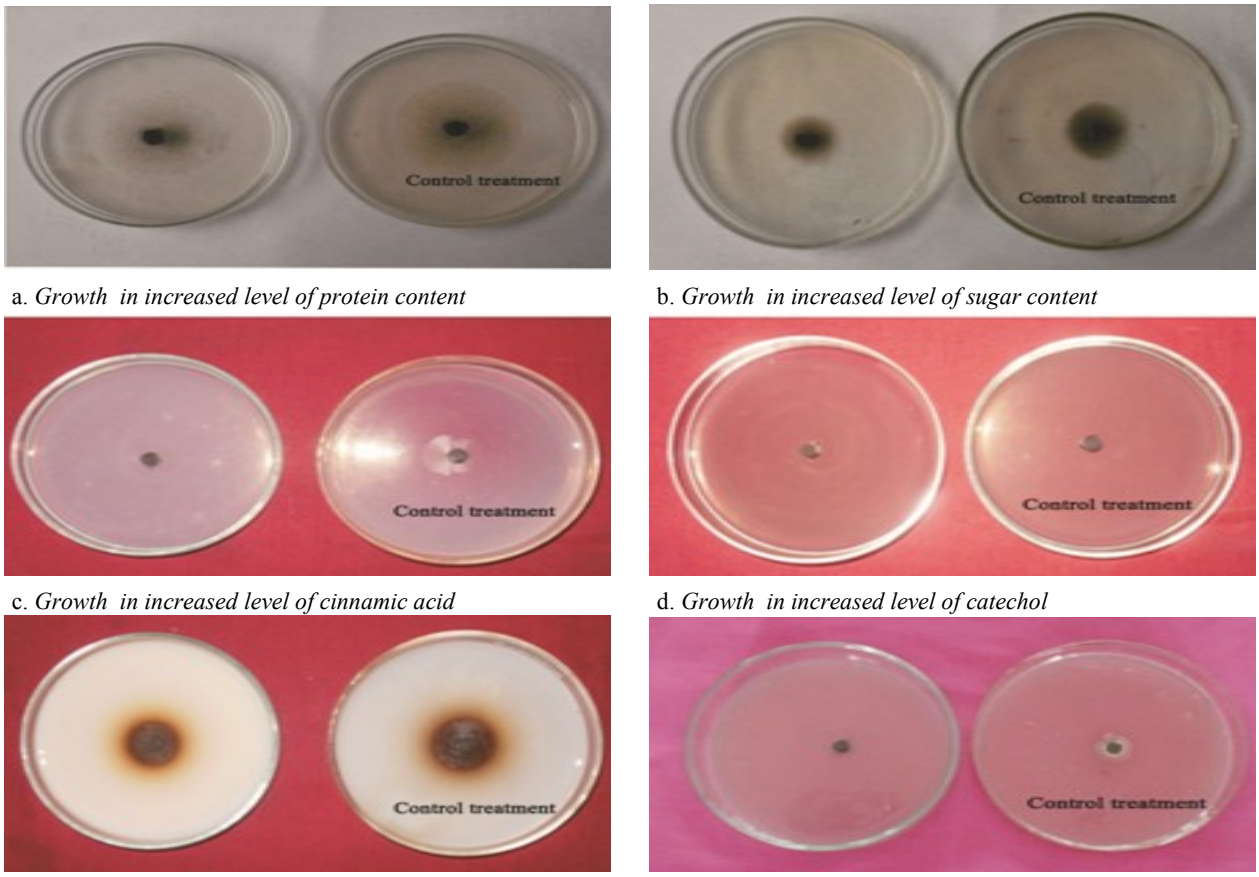
Table 4. *In vitro* effect of increased level of phytochemicals on tomato leaf blight pathogen *A. alternata*.

Phytochemicals of tomato plant	Representative test chemical	Concentration equivalent in healthy tomato leaves (mg) in test agar media	Average growth diameter (mm) of *A. alternata*	Concentration equivalent in RIC treated leaves (mg) in test agar media	Average growth diameter (mm) of *A. alternata*	Per cent inhibition of fungal growth due to respective biochemical
Protein	Serum albumin	5.702	44.00	10.087	44.00	0.00
Sugar	Glucose	2.202	22.33	4.461	18.00	19.39
Phenol	Cinnamic acid	6.917	18.33	9.643	0.00	100.00
	Catechol	0.233	0.00	0.397	0.00	100.00
	Tannic acid	0.233	21.67	0.397	18.67	13.84
	Sugar+phenols (cinnamic acid +catechol)	-	12.23	-	0.00	100.0

seedling were treated with resistance inducing chemicals. The maximum increase was observed for salicylic acid @ 1.5 mM concentration which was 61.38 per cent more over control. The increase in phenol content was statistically significant for seed, seedling and seed+seedling treatment with resistance inducing chemicals over the control.

***In vitro* effect of increased quantities of protein, sugar and phenolic compounds on leaf spot pathogen:** The results (Table 4) indicated that the increased level of protein content (equivalent to increased protein in tomato leaves due to RI treatment) has no effect on inhibition of *A. alternata* fungal growth. However the increased level of sugar (equivalent to increased sugar due to RI in tomato leaves) inhibited the average growth of *Alternaria* to the tune of 19.39 per cent. Among phenolic compounds catechol was found completely inhibitory to the *Alternaria* fungus and there was no growth in catechol concentration equivalent to in tomato leaves and RI treated leaves. Cinnamic acid (at equivalent concentration in tomato leaves) had supported the growth of *Alternaria* whereas the increased level of cinnamic acid (due to RI treatment of tomato plant) had completely inhibited the growth of *Alternaria* fungus. The results are indicative that the phenolic compound catechol as such and the cinnamic acid was inhibitory to the *Alternaria* fungal pathogen of tomato. The phenolic compound tannic acid (with increased concentration due to resistance inducing chemical in tomato leaves) had some effect on inhibiton of *Alternaria* growth (13.84 % fungal growth inhibition). When the increased level of sugar + phenols (equivqlent in tomato leavesdue to RI treatment) was tested against the pathogen, it had completely inhibited the growth of *Alternaria* fungus. These results are indicative that resistance inducers increase the pathogen inhibitory biochemicals particularly sugar and phenol in tomato leaves to restrict the growth of pathogen and thereby confirm the resistance/ induce resistance in the tomato plant.

Samia and Khallal (2007) sprayed the *Fusarium oxysporum* infected tomato plants (three times) with inducer (JA and SA) and showed that total soluble sugars, free amino acids and total soluble proteins increased in both leaves and roots of JA& SA- treated plants as compared with infected control. Barilli *et al.* (2009) reported production of phenolic compounds such as scopoletin and pisatin by use of resistance inducing chemical benzothiadiazole, was inhibitory to the fungal pathogen of pea rust at early stage. These compounds showed a similar inhibitory effect when exogenously applied *in vitro* bioassay. Ashry and



a. *Growth in increased level of protein content*

b. *Growth in increased level of sugar content*

c. *Growth in increased level of cinnamic acid*

d. *Growth in increased level of catechol*

e. *Growth in increased level of tannic acid*

f. *Growth in increased level of sugar + phenols*

Fig. 1. *In vitro* effect of increased level of biochemical constituents on growth of *A. alternate.*

Mohammad (2012) reported the higher content of total phenol in resistant flax line than susceptible one and this higher level of phenol was inhibitory to the pathogen.

Flors *et al.* (2004) demonstrated the effect of FGA (Furfuryl amine; 1,2,3,4 tetra-O-acetyl-β-glucose; adipic acid mono ethyl ester) as antimicrobial activity. They found that FGA reduced the growth of filamentous fungi *A. solani* and *Botrytis cinerea* and the oomycetes *Phytophthora capsici* and *P. infestans in vitro*. Experiments on *B. cinerea* and *A. solani* indicated that this compound prevented spore germination in addition to mycelia growth. Wu *et al.* (2010) evaluated the *in vitro* effect of an externally supplied tannic acid on soil borne pathogen *Fusarium oxysporum* f.sp. *nivum*. Their results showed that the tannic acid decreased the growth of the fungus up to 9.5 % at 800 mgl^{-1}. Conidial germination was reduced by 52.3 % in comparison with the control. Maddox *et al.* (2010) evaluated 12 phenolic compounds, representing phenolic acid, coumarin, stibene and flavonoid against *Xylella fastidiosa* which cause diseases to many crop species using *in vitro* agar dilution assay. These phenolic compounds particularly catechol, caffeic acid and resvertrol showed strong anti *Xylella* activities. Ashry and Mohammad (2012) reported higher content of total phenol in resistant flax line than susceptible one and this higher level of phenol was inhibitory to the pathogen. Moghaddam and Ende (2012) reported that sugars were involved in many metabolic and signalling pathways in plants. Sugar signals may also contribute to immune responses against pathogens and probably function as priming molecules leading to pathogen-associated molecular patterns (PAMP)-triggered immunity and effector-triggered immunity in plants. Thus increased level of sugar and phenol, not only inhibit the pathogen in host system but also trigger the immunity in plant due to accumulation PR-proteins.

Conclusion

The increase in the quantity of soluble proteins and phenols in tomato leaves were maximum due to seed+seedling treatment of salicylic acid @ 1.5 mM concentration with 76.90 per cent and 102.68 per cent over control respectively whereas reducing sugar was maximum for seed+seedling treatment of β-aminobutyric acid @ 15.0 mM concentration with 61.38 per cent increase over control. Among the increased level of these biochemicals, the increased level of sugar+phenol tested against the pathogen completely inhibited the growth of *Alternaria* fungus. Thus, the increased level of reducing sugar and phenol in tomato leaves due to the application of resistance inducing chemicals seems to be inhibitory to the pathogens multiplication and pathogenesis.

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