

Inductions of Defense Response in Tomato against Fusarium Wilt through Inorganic Chemicals as Inducers

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Abstract

Pre-foliar spray with indole acetic acid, metalaxyl, dipotassium hydrogen orthophosphate, hydrogen peroxide, calcium chloride, salicylic acid and ferric chloride as inducers provided induced resistance in plant against *F. o. f.sp. lycopersici*, resulting in a decline in the disease incidence from 90.96 to 9.30% after 15 days of pathogen inoculation. The minimum disease incidence (9.30%) was reported from calcium chloride treated plants. Challenge inoculation with abiotic inducers sensitized the seedling to produce increased level of soluble protein. The maximum increase of soluble protein content was found in calcium chloride treated plant showing 35.93, 36.27 and 35.22 mg/g of fresh leaves at 5, 10 and 15 days of pathogen inoculation. Similarly, phenol content was also found to be maximum in calcium chloride treated plant, representing 2.45, 2.76 and 2.67 mg/g of fresh leaves at 5, 10 and 15 days of inoculation. Correlation coefficient analysis revealed that there was negative correlation ($r = -0.6214, -0.5867$ and -0.5484) between disease severity and soluble protein content after 5, 10 and 15 days of treatment. Similarly, total phenol content also showed negative correlation ($r = -0.5370, -0.5656$ and -0.4225) with disease incidence. Protein profiling by SDS-PAGE revealed that pre-foliar spray with calcium chloride synthesizes new proteins representing maximum number of 14 protein bands. The genomic DNA was further investigated using 5 universal ITS primers alone and in combination with each other as forward and reverse of Genei series showing all monomorphic bands at 350 bp as compared to 100 bp DNA ladder marker. All the treatments from T1 to T8 of tomato variety, Azad T6 shows the unique identification of combination with ITS primer 1 and 4.

Keywords: Biochemical changes; Fusarium wilt; Genomic DNA; Induced resistance; Protein profiling and tomato

Introduction

Tomato (*Lycopersicon esculentum* L. Krust) is considered as one of the most important and remunerative vegetable crop cultivated throughout the world owing to its high nutritive value as well as its antioxidant and curative properties. The worldwide production of tomato was about 130 million tonnes in the year 2008, China being the largest producer in the world with a production of 33.811 million tonnes. In India, the total production of tomato in the year 2008-09 was 11.149 million tonnes from 0.599 million hectare of land which is low as compared to other country. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc), is one of the major yield limiting factors in India, as described by Synder and Hansen [1]. The management of the disease can be done through cultural, chemical, biological control and use of resistant varieties. But most of the conventional chemical, biological and use of resistant varieties tend towards the direct control of the pathogen by their elimination. Sometimes, these practices raise problem due to development of resistant strains of the pathogen which may become very tedious to control. To overcome these problems, search for new areas for managing the disease are explored. One of the best strategies developed to manage various diseases is induced resistance. It has been found that pre-application of tomato seedling with bio-agents, plant extracts, avirulent races of pathogens and some inorganic chemical like phosphate salt, (dipotassium/sodium or tripotassium) silicon (SiO_2) provided the systemic induced resistance in various crops [2-9]. Biochemical and physiological changes associated with induction of resistance are due to the response to inducing agents which are in the form like phytoalexins (Paxtron), lignin [10], callose [11] and plant pathogenesis related proteins [5]. Inducers also lead to formation of additional secondary xylem vessels in plant system [12]. These observations led to exploration in the present investigation.

Materials and Methods

Collection of diseased sample

Tomato plants with typical wilt like symptoms were first identified and then collected from Vegetable Research Farm, C.S.A. University of Agriculture and Technology, Kalyanpur (Kanpur). The diseased plants were uprooted and properly packed in polythene bags and brought to the laboratory for initial examination. The samples were pressed in between the fold of sterilized blotter paper and preserved at 4 to 6°C in refrigerator for further investigation.

Isolation of pathogen

The diseased plant with typical wilt symptom was used for isolation of the pathogen. The diseased plant's stem was taken and washed thoroughly with tap water and finally with distilled water to remove all dust particles. The diseased part of the stem was then cut into small pieces by a sterilized knife in such a way that each bit contain small amount of diseased and healthy tissues. The chopped pieces were then dipped in 0.1% HgCl_2 (Mercuric chloride) solution for 30 seconds and then thoroughly washed with distilled water thrice to remove any trace amounts of HgCl_2 solution, if present. The excess moisture was removed by placing these pieces in between two folds of sterilized blotter paper.

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The pieces were then placed in sterilized petri plate containing Potato Dextrose Agar (PDA) which was previously poured under aseptic condition. Finally, the plates were sealed with parafilm tape and were incubated at $27 \pm 1^\circ\text{C}$. The petri plates were observed daily to observe the growth of the mycelium around the bits. The pathogen was purified by hyphal tip method.

Identification of the pathogen

After purification of fungi, a slide was prepared from fungal colony and observed under compound microscope. The pathogen was identified on the basis of its morphological and cultural characteristics as described by Synder and Hansen [1].

Collection of abiotic inducers

The abiotic inducers (as mentioned earlier) were collected from local market to evaluate their effect as inducers against Fusarium wilt in tomato.

Preparation of abiotic solutions

For preparation of different concentrations of inducers (salicylic acid (SA), calcium chloride (CC), dipotassium hydrogen phosphate (DHP), hydrogen peroxide (HP), ferric chloride (FC), indole-3 acetic acid (IAA) and metalaxyl), the required quantity of inducing agents are weighed and placed in conical flask separately and 100 ml of sterilized water was added to each conical flasks and shaken until they are dissolved completely to prepare the required concentration (4 mM, 10 ppm, 10 mM, 1.0%, 5 mM, 0.2% and 0.2% respectively) of solutions.

Effect of inducers on disease severity

In order to ascertain the effect of inducing agents on disease development, an experiment was conducted in the glasshouse with three replications for each treatment. Plants were sprayed with inducers for 48 hrs before root inoculation with pathogen. After inoculation with pathogen, plants were covered with polythene bags for 48 hrs to provide suitable moisture and humidity for development of pathogen. During the course of this experiment, two controls are kept; in one case, plants were sprayed with water (Check-1) and in second case, plants were inoculated with conidial suspension of *F. o. f.sp. lycopersici* (Check-2). After 10 days of inoculation, observations were taken on disease severity.

Inoculation of pathogen

After two days of spraying, plants were inoculated with spore suspension of pathogen. The spore suspension of the pathogen was prepared from seven days old culture. The concentration of conidia was maintained as 10,00,000 conidia/ml. The homogenized spore suspension was inoculated at the base of the stem near root zone at 2 ml suspension/plants. The plants were then covered with polythene bags for 48 hrs to provide suitable moisture and humidity for growth and development of the pathogen. Two controls were kept. In one case, plants were inoculated with the pathogen which served as Control-2 and in other cases plants were sprayed with distilled water which served as Control-1.

Measurement of disease incidence

The disease incidence was measured after 10 days of pathogen inoculation. The disease severity was recorded by 0-4 scale as described by Weitang et al. where zero represents no infection and four denotes complete infection. Eight replications were maintained for each

treatment in two separate experiments. The 0-4 scale of the disease severity was classified as follows:

0 - No infection.

1 - Slight infection, which is about 25% of full scale, one or two leaves turned yellow.

2 - Moderate infection, two or three leaves turned yellow, 50% of leaves wilted.

3 - Extensive infection, all the leaves turned yellow, 75% of leaves wilted and growth is inhibited.

4 - Complete infection, the leaves of the whole plant turned yellow, 100% of leaves wilted, and the plants died.

The percentage of disease incidence was determined using the formula given by Weitang et al.

$$\text{disease incidence (\%)} = \left[\frac{(\sum \text{scale} \times \text{number of plants infected})}{(\text{highest scale} \times \text{number of plants})} \right] \times 100.$$

Biochemical analysis

Biochemical analysis of resistance response in plant due to effect of inducers: Analysis of biochemical changes in tomato plant due to foliar spray with abiotic inducers was carried out to determine the effect of different treatments on the contents of soluble protein and phenol in the plant.

Tomato leaves were collected from different treatments and the changes in the content of soluble protein and phenol in leaves were estimated at 5, 10, and 15 days after inoculation of the pathogen.

Soluble protein content: Tomato leaves were harvested from plants sprayed with different treatments. It was then washed with distilled water several times and dried on blotter paper. A quantity of 1.0 gm of each sample was cut into small pieces and ground in pestle and mortar using extraction buffer as 1:5 (w/v). The suspension was centrifuged at 10,000 rpm for 30 minutes at 4°C . The supernatant was collected and used for quantification and profiling of protein.

Quantification of protein: The method developed by Lowry et al was used with slight modification for quantification of the total soluble protein content. Standard solutions of Bovine Serum Albumin were pipetted out into a series of test tubes. Similarly, same volumes of sample extracts were also pipetted out and kept in other test tubes separately. The volume in all the tubes was made up to 1 ml with distilled water. A tube with 1 ml of distilled water served as a blank. Later on, 5 ml of alkaline copper solution was added in each test tube and incubated at room temperature for 10 minutes. Thereafter, 0.5 ml of Folin-Ciocalteu reagent (FCR) was mixed well and incubated at room temperature for 30 minutes in dark place. The absorbance at 660 nm against the blank was read. The standard graph of BSA was drawn to calculate the amount of soluble protein in different samples. Protein estimated was represented as mg/g of fresh leaf.

Total phenol estimation: The accumulation of phenols in tomato seedlings was estimated following the procedure developed by Bray and Thorpe with slight modification. In this method, the total phenols estimation was carried out with FCR, which was measured at 650 nm calorimetrically.

Exactly, 1.0 g of tomato seedlings were ground in a mortar and pestle along with 80% ethanol (1:10 w/v). It was then centrifuged at 10,000 rpm for 30 minutes at room temperature in order to homogenate the

suspension. Supernatant was separated and re-extracted with 5 times volume of 80% ethanol, centrifuged and the supernatant was pooled. It was then evaporated to dryness and residues were dissolved in 5 ml of distilled water. Different aliquots were pipetted out into test tubes and the volume in each tube was made to 3 ml with distilled water. A test tube with 3 ml distilled water served as blank. Subsequently, 0.5 ml of FCR was added and after three minutes, 2 ml of 20% Na₂CO₃ solution was thoroughly mixed in each tube. After this, the tubes were placed in boiling water for 1 min and then cooled at room temperature. Then absorbance at 650 nm against blank was measured using Ultra Violet Visible (UV-VIS) spectrophotometer and the standard curve using different concentration of catechol was prepared. From the standard curve, the concentration of phenols in the test sample was determined and expressed as mg/g of fresh sample materials.

Correlation coefficient and Regression equation

The biochemical analysis of tomato leaves under different treatments and disease incidence of the corresponding value under an experiment was done to determine the level of correlation coefficients (r) between soluble protein and disease incidence as well as between total phenol and disease incidence. Simple regression equations ($Y=a+bx$) were also developed for both the variables (Protein and Phenol) separately to understand their relation with disease incidence.

Protein profiling

Profiling of soluble proteins was also done in various treatments. Analysis of total soluble proteins through Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out for the study of variable response of resistance to *Fusarium oxysporum* f.sp. *lycopersici*. SDS PAGE was done to get soluble protein pattern. Soluble proteins were electrophoresed by 12 percent SDS polyacrylamide gel based on the method of Laemmli [13].

Isolation and purification of total genomic DNA

Isolation of DNA: Genomic DNA from seedlings (treated samples) was isolated from all the treatments as described by Doyle and Doyle [14] with slight modification.

Five grams of tomato leaves were harvested and quickly ground in 15 ml of grinding buffer with the help of mortar and pestle. The resulting paste was transferred to a 50 ml centrifuge tube and incubated at 65°C in water bath for 30 minutes. After 30 minutes, 3 ml of 10 M ammonium acetate was added and again incubated for 16 minutes. It was then centrifuged at 10,000 rpm for 10 minutes at 18°C. Three layers were formed. The supernatant was collected into a clean 30 ml tube and equal volume of chilled isopropanol was added and kept at -20°C overnight. The DNA precipitated out as a pellet at 10,000 rpm for 10 minutes, washed with 70% ethanol, dried and dissolved in 1.0 ml of TE buffer. The solution was transferred to eppendorf tube and equal volumes of Phenol, Chloroform and Isoamyl alcohol (25:24:1) were added and gently mixed. The mixture was centrifuged at 10,000 rpm for 5 minutes and supernatant was transferred to a fresh tube. Extraction was repeated twice with equal volumes of Chloroform and Isoamyl alcohol (24:1). Two volume of chilled ethanol was added and kept at -20°C for 1 hour. The DNA, thus precipitated out, and was washed with 70% ethanol, dried and dissolved in 1 ml T₁₀ E₁ buffer.

Purification of genomic DNA: After dissolving the DNA in TE buffer, 10-15 µl of RNase A (10 mg/ml) per 500 µl of DNA sample was added and incubated at 37°C for 1 hour to degrade RNA. Equal volumes of 25:24:1 Phenol:Chloroform:Isoamyl alcohol mixture

(pH 8) was added, gently mixed for 5 minutes and centrifuged for 10 minutes at 10,000 rpm. Three layers were formed, the upper layer was separated into a 2 ml eppendorf tube and double volume of 100% chilled ethanol was added for DNA precipitation (DNA was seen as clumped). Precipitated DNA was then centrifuged at 10,000 rpm for 10 minutes at room temperature and the supernatant was discarded. For easy pelleting, 3 N sodium acetate or 5 M ammonium acetate at 1/10 of DNA volume was added. The pelleted DNA was washed thrice in 70% ethanol with gentle tapping to expel salts. Pellets were dried and dissolved in 100-200 µl T₁₀ E₁ buffer.

Quantification of genomic DNA: Quantification of DNA was accomplished by analyzing the purified DNA on 0.8% agarose gel alongside uncut lambda DNA as standard. DNA was diluted in T10 E1 buffer to a concentration of approximately 12.5 ng/µl for use in PCR analysis. Yield of purified DNA was also estimated by taking the absorbance at 260 nm, using Genosys 6 spectrophotometer. To calculate the yield of DNA, the relationship 1 A 260 unit = 50 µg double stranded DNA was used.

PCR condition: The PCR conditions included initial denaturation of DNA at 94°C for 2 min followed by 41 cycles of DNA template denaturation at 92°C for 1 min, primer annealing at 40°C/42°C/45°C/45°C/53°C, respectively for 1 min, DNA amplification at 72°C for 3 minutes and final primer extension at 72°C for 10 min.

Agarose Gel Electrophoresis: The amplified products were separated by electrophoresis in 1.20% agarose gel containing ethidium bromide (10 mg/ml) using 1X TBE buffer at 50-60 volt/cm for three hours. The size of amplified fragments was determined by comparison with standard molecular weight markers (100 bp DNA ladder) DNA fragments were visualized in UV light and photographed using gel documentation system for permanent records. The amplification product is stored at 40°C till electrophoretic separation.

Results

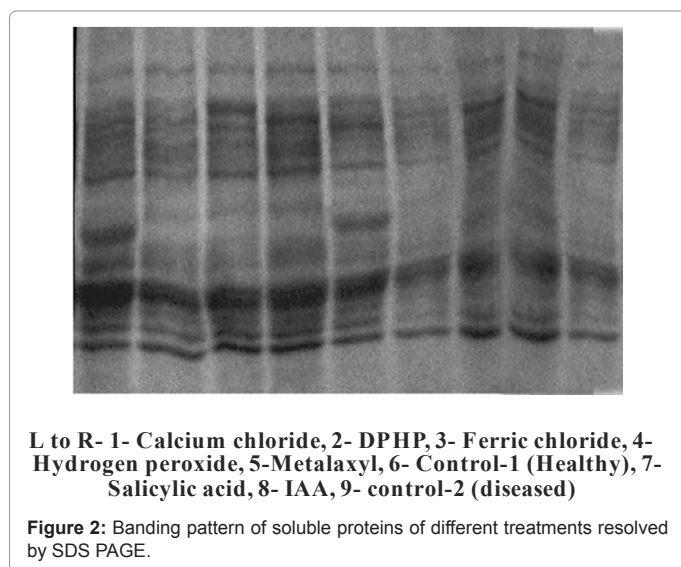
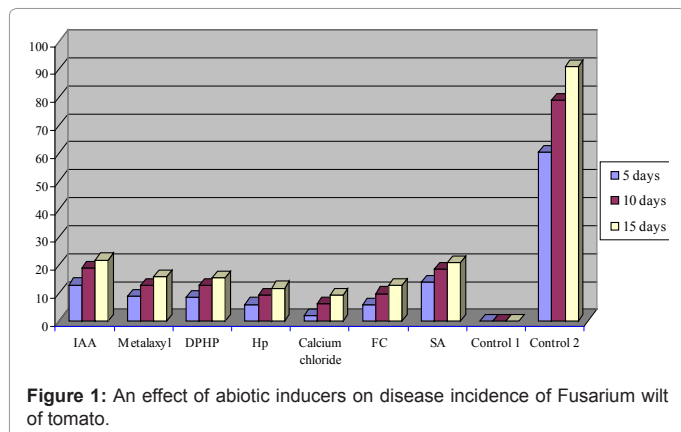
The present investigation was undertaken to evaluate the potential of various inorganic chemicals i.e. IAA, calcium chloride, metalaxyl, DPHP, hydrogen peroxide, salicylic acid, ferric chloride, as inducers in induced resistance in tomato against the Fusarium wilt caused by *F. o. f.sp. lycopersici*. Biochemical changes in association of induction of resistance in tomato plants after the application of abiotic inducers (inorganic chemicals) were assessed. Results of the experiments are presented below.

Effect of treatment with chemical on development of disease

The effect of pre-foliar spray of abiotic inducers on tomato plants revealed that there is a decline in wilt incidence due to various treatments under glasshouse condition (Figure 1). The susceptible variety, Azad T-6 of tomato showed 100% wilt incidence in case of *F. o. f.sp. lycopersici* treated plants. The minimum wilt incidence was recorded in calcium chloride treated plants which was 2.10% followed by ferric chloride, hydrogen peroxide and DPHP treated plants which was 5.98%, 6.12% and 8.54% disease incidence, respectively. The decrease in disease incidence might be the activities of inorganic chemicals, which act as inducers in inducing resistance in plants against *F.o. f.sp. lycopersici*.

Biochemical changes associated with foliar spray of chemicals

To evaluate the biochemical changes associated with foliar spray, one month old plants were sprayed with solutions of abiotic inducers. The pathogen was inoculated after 48 hours of foliar spray. The soluble



protein and total phenol content were estimated at 5 days, 10 days and 15 days of pathogen inoculation.

Total soluble protein

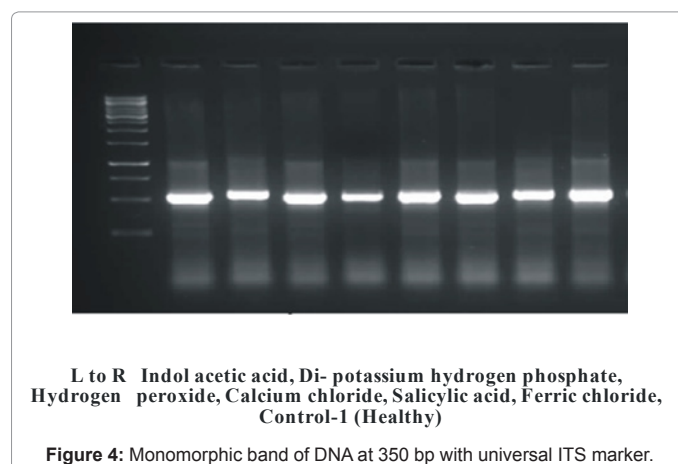
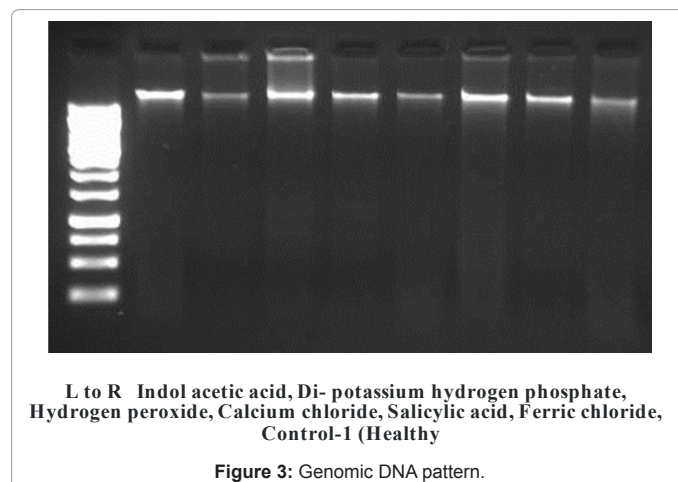
The result presented in the Table 1 indicate that the soluble protein contents in calcium chloride treated leaves were 35.93 mg/g, 36.27 mg/g and 35.22 mg/g of fresh leaves at 5, 10 and 15 days of pathogen inoculation which is the highest among all the treatments. The soluble protein contents in control-1 plants were 21.14 mg/g, 22.60 mg/g and 21.21 mg/g at 5, 10 and 15 days after pathogen inoculation whereas in case of control-2, the values are 17.74 mg/g 19.69 mg/g and 19.12 mg/g, respectively. The calcium chloride treated leaves had 60.48% higher protein content over control-1 and 84.20% higher than control-2 at 10 days of pathogen inoculation. Other treatments like hydrogen peroxide (35.24 mg/g), ferric chloride (34.19 mg/g), and DPHP (33.73 mg/g) increased the protein content by 55.92%, 51.28%, and 49.24%, respectively over control-1 and 78.97%, 73.64%, and 71.30% over control-2. The rest of the treatments also had significantly higher protein content than control-1 and control-2. From the Table 2, it is also clear that all treatments increased protein content to a maximum at 10th day of pathogen inoculation, thereafter, it was decreased gradually from 10 to 15 days. The increased protein in treated plants might be responsible for defense response in plants.

Total phenol

The result presented in Table 3 shows that all the treatment significantly increased the total phenol content as compared to control-1 and control-2 at 5, 10, and 15 days of pathogen inoculation. The maximum amount of phenol content was found in calcium chloride treatment with a value of 2.45 mg/g, 2.76 mg/g and 2.67 mg/g of leaves against 1.56 mg/g, 1.76 mg/g and 1.71 mg/g in case of control-1 and 1.47 mg/g, 1.52 mg/g and 1.44 mg/g in case of control-2 at 5th, 10th and 15th days of pathogen inoculation. The percent increase in phenol content in calcium chloride treated leaves were 56.81% and 81.57% higher than control-1 and control-2, respectively at 10th day of pathogen inoculation followed by hydrogen peroxide, ferric chloride and DPHP. The data also shows that the phenol content for all treatments increase from 5 to 10 days period but again decreases from 10 to 15 days. The result summarized that the phenol content in all treated plants increased up to a certain period and thereafter, it was decreased. The increased phenol content in treated plants might be responsible for defense response in plant against *F. o. f.sp. lycopersici*.

Protein profiling

Protein profiling of soluble protein from fresh tomato leaves was done to determine whether any new protein was associated with resistance to *F. o. f.sp. lycopersici* in tomato (variety Azad T6) or not due to various chemicals as inducers. SDS PAGE was used for finding the banding patterns of proteins. The banding patterns of soluble



Sl.No.	Treatment	Total soluble protein content mg/g fresh leaves			% increase	
		5 days	10 days	15 days	Over control-1	Over control-2
1.	Indol acetic acid	27.33	27.51	26.54	21.72	39.71
2.	Metalaxyl	31.79	32.23	31.26	42.61	63.68
3.	Diammonium potassium orthophosphate	33.14	33.73	32.64	49.24	71.30
4.	Hydrogen peroxide	34.71	35.24	33.94	55.92	78.97
5.	Calcium chloride	35.93	36.27	35.22	60.48	84.20
6.	Salicylic acid	28.37	29.11	28.14	37.65	47.84
7.	Ferric chloride	33.71	34.19	33.29	51.28	73.64
8.	Control-1 (Healthy)	21.14	22.60	21.21		
9.	Control-2 (Diseased)	17.74	19.69	19.12		
	S.E. (Diff)	0.07459	0.13501	0.11159		
	C.D.(P= 0.05)	0.15670	0.28364	0.23444		

Table 1: Effect of foliar spray with abiotic inducers (inorganic chemicals) on total soluble protein content of tomato leaves after 5 days, 10 days and 15 days of pathogen inoculation.

Sl.No.	Treatment	Total phenol content mg/g fresh leaves			% increase	
		5 days	10 days	15 days	Over control-1	Over control-2
1.	Indole acetic acid	1.97	2.11	2.05	19.88	38.81
2.	Metalaxyl	2.01	2.19	2.13	24.43	44.07
3.	Diammonium potassium hydrophosphate	2.21	2.36	2.28	34.09	55.26
4.	Hydrogen peroxide	2.33	2.64	2.58	50.00	73.68
5.	Calcium chloride	2.45	2.76	2.67	56.81	81.57
6.	Salicylic acid	1.90	2.04	1.95	15.90	34.21
7.	Ferric chloride	2.29	2.56	2.46	45.45	68.42
8.	Control-1 (Healthy)	1.56	1.76	1.71		
9.	Control-2 (Diseased)	1.47	1.52	1.44		
	S.E. (Diff)	0.02867	0.03725	0.03801		
	C.D.(P=0.05)	0.06024	0.07826	0.07985		

Table 2: Effect of foliar spray with abiotic inducers (inorganic chemicals) on total phenol content of tomato leaves after 5 days, 10 days and 15 days of pathogen inoculation.

proteins from different treatments are shown in Figure 2. The number of protein bands present in each treatment range from 14 to 7. The maximum number of bands is obtained from calcium chloride treated plants followed by hydrogen peroxide, ferric chloride and DPHP and minimum number of bands were found in diseased untreated plants. The banding pattern of proteins from figure represent that some new protein is synthesized in calcium chloride treated plants which were not found in any other treatment. Similarly, some new protein bands were also found in hydrogen peroxide, ferric chloride and DPHP treated plants showing 12 in each, respectively. The presence or absence of protein bands might be due to the activities of abiotic inducers in plant which may also be key factor for defense mechanism in tomato against *F. o. f.sp. lycopersici*.

Genomic DNA

The genomic (Figure 3) DNA of tomato Azad T6 variety has been isolated from tomato seedlings treated with abiotic inducers by using Doyle and Doyle protocol [14]. The DNA was further purified by Phenol-Chloroform-Isoamyl Alcohol (PCI) and RNase treatment followed by DNA amplification with PCR. The genomic DNA was also quantified by using spectrophotometer & visualized in 0.8% agarose gel. In the present investigation, 5 universal ITS primers (Table 4) are used alone and in combination with each other as forward/reverse of gene series showing all monomorphic bands at 350 bp as compared to 100 bp DNA ladder marker. All the treatment from T1 to T 8 of tomato variety Azad T6 shows the unique identification of combination with ITS primer 1 & 4 as shown in Figure 4 while remaining alone and

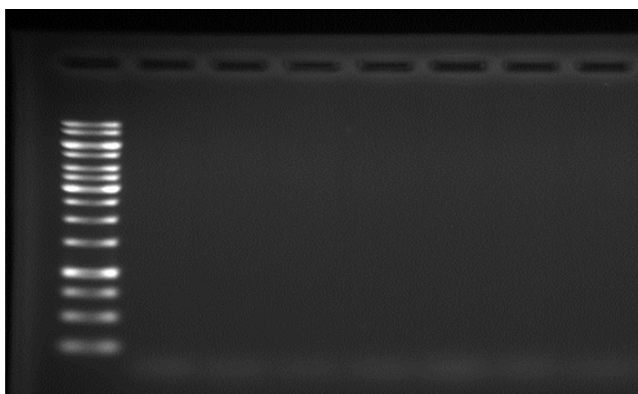
in combination of ITS primer does not show any monomorphism, hence the gel is totally blank as shown in Figure 5. Therefore, it may be concluded that ITS 1 as a forward and ITS 4 as reverse sequences has the uniqueness in the tomato variety Azad T6 which can be further used for identification of Azad T₆ variety of tomato G.

Correlation coefficient and regression equation

The leaves treated with inorganic chemicals as inducers in defense response showed decreased disease incidence with increased level of soluble protein. A negative correlation (r), -0.6214, -0.5867 and -0.5484 was found between disease incidence and soluble protein content. Similarly, diseased incidence decreased with increased level of total phenol content and there was also a negative correlation (r) -0.5370, -0.5656 and -0.4225 between total phenol content and disease incidence. The corresponding simple regression equation also showed the negative correlation between total soluble protein and disease incidence as well as total phenol and disease incidence (Table 3, Figure 6 and 7).

Discussion

Induced resistance prior to challenge infection elevates the level of some defense compounds and sensitize the plants to rapidly produce some compounds after infection and thereby, provide protection against the disease (Kuc). The induced resistance in cucumber against *C. lagenarium* by Ciba-Geigy compound (CGA-41396) was attributed to the major accumulations of protein including chitinase and chitinase mRNA [15]. Lorenzo et al also reported that when wheat leaves were



L to R Indol acetic acid, Di-potassium hydrogen phosphate, Hydrogen peroxide, Calcium chloride, Salicylic acid, Ferric chloride, Control-1 (Healthy)

Figure 5: Blank gel with no amplification with ITS marker.

Biochemical parameters	Days after pathogen inoculation	Correlation coefficient (r) with disease incidence	Regression equation
Total soluble protein	5 days	-0.6214	Y=32.2247-0.2143X
	10 days	-0.5867	Y=32.7956-0.1462X
	15 days	-0.5484	Y=31.6708-0.1186X
Total phenol	5 days	-0.5370	Y=2.1532-0.0100X
	10 days	-0.5656	Y=2.4014-0.0099X
	15 days	-0.4225	Y=2.2224-0.0087X

Table 3: Correlation of disease incidence with total soluble protein and total phenol content of tomato leaves.

S.No		Sequence (5'-3')
1.	ITS-1	TCC GTA GGT GAA CCT GCG G
2.	ITS-2	GCT GCG TTC TTC ATC GAT GC
3.	ITS-3	GCA TCG ATG AAG AAC GCA GC
4.	ITS-4	TCC TCC GCT TAT TGA TAT GC
5.	ITS-5	GGA AGT AAA AGT CGT AAC AAG G

Table 4: Lists of ITS (universal) primer used in the study.

treated with kinetin, the synthesis of protein was higher in treated leaves than untreated leaves. Biswas et al. [16] reported that pre-inoculation sprays of crude extract sensitized seedlings to produce elevated levels of protein, soluble protein and phenol contents. Bonhamou [17] earlier also have demonstrated the use of chitosan with an endophytic bacterial strain for disease resistance against Fusarium wilt.

Phenols are well known antifungal, antibacterial and antiviral compounds. The phytoalexins involved in disease resistance are phenols in chemical constitution. Phenols are involved in disease resistance in many ways like hypersensitive cell death or lignifications of cell walls [18-20]. Biehn et al. [21] found that increased synthesis of phenols in response to inoculation with *H. carbonum* race in soybean and the level of phenols was 4 to 5 times increased over healthy plants after 24 hours of inoculation. Vidhyasekaran et al. [22] also reported enhanced quantity of phenols in resistant ragi plants to *H. tetramera*. Sivakumar and Sharma [23] expressed the view that increase in phenolic content in maize leaf sheaths inoculated with *R. solani* or plants raised from *P. fluorescens* treated seeds.

The negative correlation co-efficient between soluble protein and

total phenol with disease incidence were also found in rice against brown leaf spot [16], in wheat against spot blotch [24].

The profiling of soluble protein by SDS-PAGE revealed the qualitative and quantitative differences on comparing the pattern of soluble proteins with standard among the treatments. Biswas et al. (2003) reported that some new proteins were associated with resistance to *Bipolaris sorokiniana* induced by crude extracts of *Chaetomium globosum*. They found that some new proteins of 110 kDa, 105 kDa, 32 kDa, 35 kDa and 38 kDa were resolved by SDS PAGE analysis. These proteins were missing in unchallenged healthy, diseased seedlings and in some other treatments. The protein of 110 kDa was conspicuously not tagged in various treatments except pre-inoculation with crude extracts of *Chaetomium globosum*. The possible role of this protein for induction of resistance was speculated. Alfano et al. [25] considered that PR-proteins are involved with defense in plants to pathogens. Induction of systemic resistance in tobacco after inoculation with *Pseudomonas tabaci* was followed by an increase in concentration of

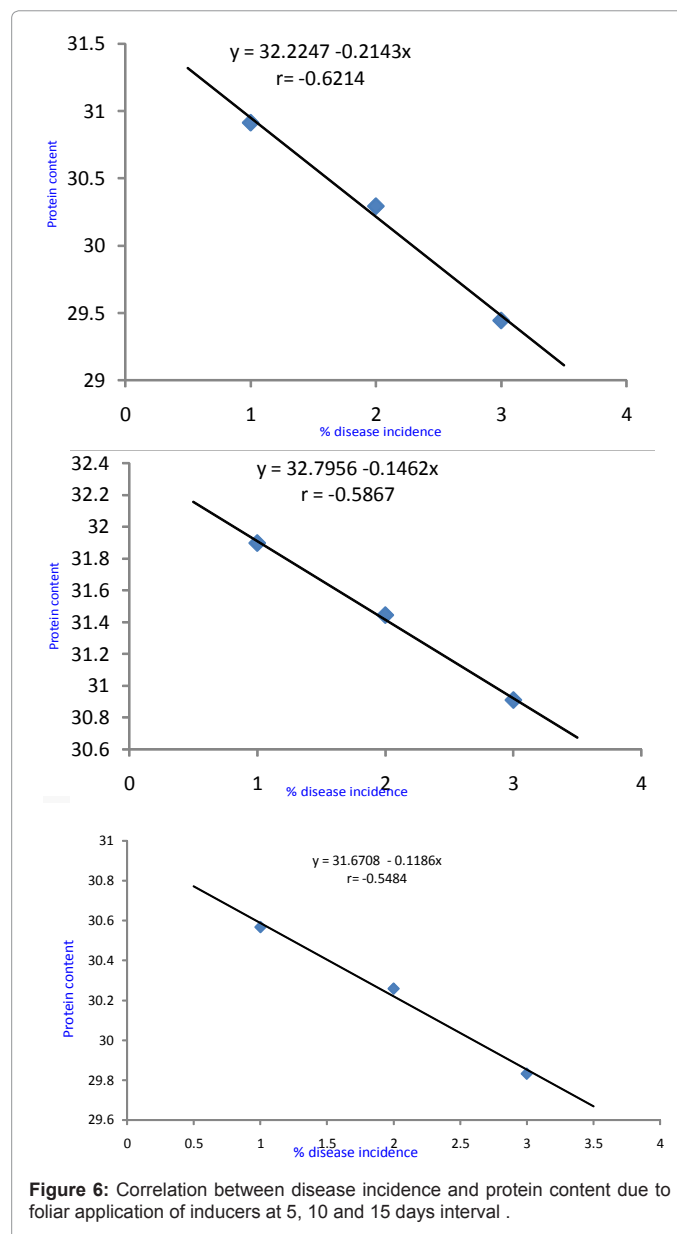
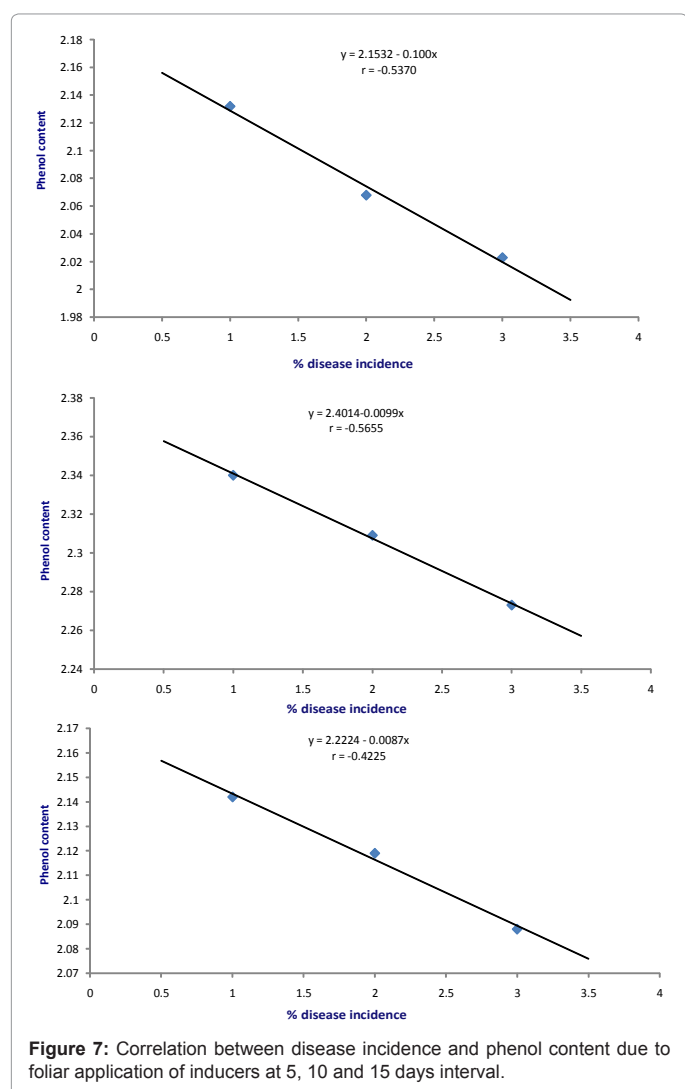


Figure 6: Correlation between disease incidence and protein content due to foliar application of inducers at 5, 10 and 15 days interval.



PR proteins [26]. A 23 kDa protein was detected in leaves of tobacco which was previously immunized with TMV [27].

Alfano et al. [25] identified 45 genes to be differentially expressed across the replicated treatments and 41 of these genes could be assigned to at least one of seven functional categories. *T. hamatum* 382-induced genes have functions associated with biotic or abiotic stress, as well as RNA, DNA and protein metabolism.

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