academicJournals

Vol. 9(1), pp. 47-52, 7 January, 2015
DOI: 10.5897/AJMR2014.7222
Article Number: D3829A250030
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Induction of defense response in Indian mustard against Alternaria blight through abiotic inducers

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Received 28 October, 2014; Accepted 12 December, 2014

Soaking seed overnight and foliar spray with salicylic acid, indole acetic acid, metalaxyl, dipotassium hydrogen orthophosphate, hydrogen peroxide, calcium chloride and ferric chloride as inducers provided induced resistance in plant against *Alternaria brassicae*, resulting in a decline in the disease incidence after 10 days of pathogen inoculation. The minimum disease incidence (14%) was recorded from salicylic acid treated plants. Challenge inoculation with abiotic inducers sensitized the seedlings to produce increased level of soluble proteins. The maximum increase of soluble protein content (24.26 and 24.72 mg/g of fresh leaves) was found in salicylic acid treated plant at 5 and 10 days after pathogen inoculation. Similarly, phenol content was (24.26 and 24.72 mg/g of fresh leaves) also found to be maximum in salicylic acid treated plant at 5 and 10 days of inoculation. Protein profiling by SDS-PAGE revealed that foliar spray with salicylic acid induced the synthesis of new proteins; however, such new proteins were not recorded in control.

Key words: Alternaria blight, biochemical changes, chemical inducers, induced resistance, protein profiling, mustard.

INTRODUCTION

Rapeseed and mustard are considered as one of the most important and remunerative oilseed crop cultivated throughout the world, playing a vital role in human nutrition and oilseed economy. India is the third largest producer of rapeseed-mustard in the world after China and Canada. India has about 6.9 million ha area, 8.1 million tonnes of production annually with an average yield of 1185 kg/ha in 2012-2013 which was far below the international standard (1631 kg/ha).

Alternaria blight caused by *Alternaria brassicae* (Berk.) Sacc. is one of the major yield limiting factor in India. Management of the disease can be done through cultural, chemical, biological approaches and through the use of resistant varieties. But most of the conventional pesticides, biological agents and resistant varieties tend towards the direct control of the pathogen by their

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elimination. Sometimes, these practices create problem by developing resistant strains of the pathogen. To overcome these problems, new methods for managing the disease were explored. One of the best strategies to manage various diseases is by inducing resistance in the host plants. It has been found that pre-application of tomato seedling with bio-agents, plant extracts, avirulent races of pathogens and with some inorganic chemical like phosphate salt, (dipotassium/sodium or tripotassium) silicon triggered the induced systemic resistance in various crops (Attitalla et al., 2001; Baysal et al., 2002; Fuchs et al., 1997; Larkin and Fravel, 1999; Ramamorthy et al., 2002; Singh et al., 1990).

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 (Brown, 1964), callose (Hinch and Clark, 1982) and plant pathogenesis related proteins (Van Loon et al., 1998). Inducers also lead to formation of additional secondary xylem vessels in plant system (De Cal et al., 2000). These observations led to exploration in the present investigation.

MATERIALS AND METHODS

Isolation of pathogen

The pathogen was isolated from the leaf spots and lesions, showing the initial and conspicuous characteristic symptoms of Alternaria blight on potato-dextrose-agar (PDA) medium. The pathogen was purified by single spore method (Hansen, 1926). The pathogen was identified on the basis of its morphological and cultural characteristics.

Preparation of chemical stimulants solutions

The solutions of different inducers with different concentrations viz. salicylic acid (10 mM), hydrogen peroxide (1.0%), indole-3 acetic acid (0.2%), dipotassium hydrogen phosphate (10 mM), ferric chloride (5 mM), calcium chloride (10 ppm) and metalaxyl (0.2%) were prepared in sterile distilled water as done by Biswas et al. (2012).

Effect of inducers on disease severity

In order to study the effect of inducing agents on disease development, an experiment was conducted in glasshouse with three replications for each treatment. Three plants were maintained in each replication. Plants were sprayed with inducers at disease initiation stage, that is, after 45 days of sowing. The treated plants were challenged with the pathogen after 48 h after application. For this purpose, a spore suspension containing 10^5 conidia/ml of the pathogen was prepared from seven days old culture. The homogenized spore suspension was sprayed over the test plants with the help of automizer. After inoculation, the plants were covered with polythene bags for 48 h to provide suitable moisture and humidity for symptom development. Two types of controls were maintained. In the first case plants were sprayed with water (Check-1) and in second group plants were inoculated with conidial suspension of *A. brassicae* (Check-2). The disease severity was recorded after 10 and 20 days of inoculation. The experiment was repeated for confirmation.

Table 1. The 0–5 scale of the disease severity was classified as follows.

Grade	Infection (%)	Reaction
0	Nil	Immune (I)
1	Upto 5% infection	Resistant (R)
2	Upto 10% infection	Moderately resistant (MR)
3	Upto 20% infection	Moderately susceptible (MS)
4	Upto 30% infection	Susceptible (S)
5	40% or more	Highly susceptible (HS)

Measurement of disease incidence

The disease incidence was measured after 10 days of pathogen inoculation. The disease severity was recorded by 0-5 scale as described by Conn et al. (1990). Where zero represents no infection and five denotes complete infection. The 0-5 scale of the disease severity was classified as shown in Table 1

The percentage disease index was calculated by the following formula.

$$\text{Disease index (\%)} = \frac{\text{Sum of numerical ratings}}{\text{Total number of leaves examined} \times \text{maximum grade}} \times 100$$

Biochemical analysis

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

Soluble protein content

Mustard leaves (1.0 g) after harvest were thoroughly washed with distilled water, dried on blotter paper, cut into small pieces and grounded with pestle and mortar in extraction buffer (1:5 w/v). The suspension was centrifuged at 10,000 rpm for 15 min 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. (1951) was used. 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 min. Thereafter, 0.5 ml of Folin-Ciocalteu reagent (FCR) was mixed well and incubated at room temperature (25±2°C) for 30 min 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 was represented as mg/g of fresh leaf.

Table 2. Effect of abiotic inducers on disease severity after 10 and 20 days of pathogen inoculation.

Treatment	Disease severity after pathogen inoculation		disease control (%)	
	Disease severity after 10 days	Disease severity after 20 days	At 10 days	At 20 days
Ferric chloride ($FeCl_2$)	19.60 (26.26)	23.01 (28.62)	29.00	52.00
Hydrogen peroxide (H_2O_2)	15.48 (23.14)	17.68 (24.83)	56.37	67.09
Salicylic acid (SA)	10.45 (18.77)	14.00 (21.93)	43.91	63.13
Calcium chloride ($CaCl_2$)	12.04 (20.23)	15.78 (23.33)	41.68	58.76
Metalaxyl	16.10 (23.60)	19.77 (26.37)	62.15	70.80
Indole-3 acetic acid (IAA)	19.45 (26.14)	23.93 (29.25)	29.54	50.09
Di potasium hydrogen phosphate (DPHP)	21.42 (27.56)	25.46 (30.28)	22.38	46.91
Control 1 (healthy)	1.63 (7.11)	7.56 (15.88)		
Control 2 (infected)	27.60 (31.68)	47.95 (43.82)		
SE	1.09	1.21		
CD at 5%	3.24	3.61		

The data in the parenthesis are transformed values (angular transformation).

Total phenol estimation

The accumulation of phenols in mustard leaves was estimated following the procedure developed by Bray and Thorpe (1954). In this method, the total phenols estimation was carried out with FCR, which was measured at 650 nm calorimetrically. Leaves (1.0 g) of the treated plants were grounded with a mortar and pestle in 80% ethanol (1:10 w/v). It was then centrifuged at 10,000 rpm for 15 min at room temperature (25±2°C). 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 Na_2CO_3 (20%) solution was thoroughly mixed in each tube. After this, the tubes were placed in boiling water for 1 min and cooled at room temperature (25±2°C). The 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 samples was determined and expressed as mg/g of fresh sample materials.

Protein profiling

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was carried out to study the soluble protein profile of the challenged mustard leaves after various treatments. Soluble proteins were electrophoresed by 10% SDS polyacrylamide gel based on the method of Laemmli (1970).

RESULTS

Effect of abiotic inducers on disease development

The effect of pre-foliar spray of abiotic inducers on mustard leaves revealed that there is a decline in the disease incidence due to various treatments under glasshouse condition (Table 2). The susceptible variety of mustard, Varuna, showed maximum Alternaria blight incidence in the case of control-2. The minimum disease incidence (14.0%) was recorded with salicylic acid treated plants followed by calcium chloride, hydrogen peroxide and metalaxyl which was 15.78, 17.68 and 19.77%, respectively. The reduced disease incidence seems to be due to the various treatments that induced resistance against *A. brassicae.*

Biochemical changes associated with foliar spray of chemicals

Total soluble protein

The results presented in Table 3 indicate that the soluble protein contents in salicylic acid treated leaves were 26.47 and 27.02 mg/g of fresh leaves at 5 and 10 days of pathogen inoculation, respectively. The content was the highest among all the treatments. The soluble protein contents in control-1 plants were 14.52 and 15.29 mg/g at 5 and 10 days, respectively, after pathogen inoculation. In the control-2, the values of the same were 13.72 and 14.35 mg/g, respectively. Other treatments like calcium chloride (26.53 mg/g), hydrogen peroxide (24.72 mg/g) and metalaxyl (23.19 mg/g) also showed significant increase in protein content over the control-1 and 2. The rest of the treatments also had significantly higher protein content than control-1 and 2. From Table 3, it is also clear that all treatments increased protein content to a maximum at 10th day of pathogen inoculation.

Total phenol

The results presented in Table 4 showed that total phenol

Table 3. Effect of abiotic inducers on total soluble protein.

Treatment	Protein content (mg/g leaf weight)	
	After 5 days of pathogen inoculation	After 10 days of pathogen inoculation
Ferric Chloride ($FeCl_2$)	21.94	22.37
Hydrogen peroxide (H_2O_2)	24.26	24.72
Salicylic acid (SA)	26.47	27.02
Calcium Chloride($CaCl_2$)	25.74	26.53
Metalaxyl	22.73	23.19
Indole-3 acetic acid (IAA)	20.75	21.29
Di Potasium hydrogen phosphate (DPHP)	19.12	19.85
Control 1 (Healthy)	14.52	15.29
Control 2 (Infected)	13.72	14.35
SE	0.23	0.17
CD at 5%	0.69	0.52

Table 4. Effect of abiotic inducers on total phenol.

Treatment	Phenol content (mg/g leaf weight)	
	After 5 days of pathogen inoculation	After 10 days of pathogen inoculation
Ferric chloride ($FeCl_2$)	3.89	4.11
Hydrogen peroxide (H_2O_2)	4.43	4.52
Salicylic acid (SA)	4.85	5.02
Calcium chloride($CaCl_2$)	4.67	4.80
Metalaxyl	4.08	4.21
Indole-3 acetic acid (IAA)	3.94	4.04
Di Potasium hydrogen phosphate (DPHP)	3.45	3.73
Control-1 (healthy)	2.08	2.47
Control- 2 (infected)	1.83	1.94
SE	0.19	0.19
CD at 5%	0.57	0.57

content was significantly increased in all the treatments as compared to control-1 and 2 at 5 and 10 days of pathogen inoculation. The maximum amount of phenol content was found in salicylic acid treatment with a value of 4.85 and 5.02 mg/g of leaves against 2.08 and 2.47 mg/g in the case of control-1 and 1.83 mg/g and 1.94 mg/g in the case of control-2 after 5 and 10 days of pathogen inoculation. The data also show that phenol content in all the treatments was somewhat higher at 10 days in comparison with 5 days after pathogen inoculation.

Protein profiling

SDS PAGE experiment showed the banding patterns of soluble proteins from different treatments as presented in Figure 1. It is clear from Figure 1 that a molecular weight of 40 KDa proteins appeared on the gel. The darkest band was obtained in salicylic acid treated plants followed by calcium chloride and H_2O_2. Protein bands with similar molecular weight were also obtained with metalaxyl, ferric chloride, IAA and DPHP treated plants. Whereas, no such band was obtained in control-1 (healthy) and control-2 (infected). The banding pattern of proteins from the figure shows that some new proteins are synthesized in all the treatments which were absent in control-1 and 2. 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 *A. brassicae*.

DISCUSSION

It is evident from the above mentioned results that in

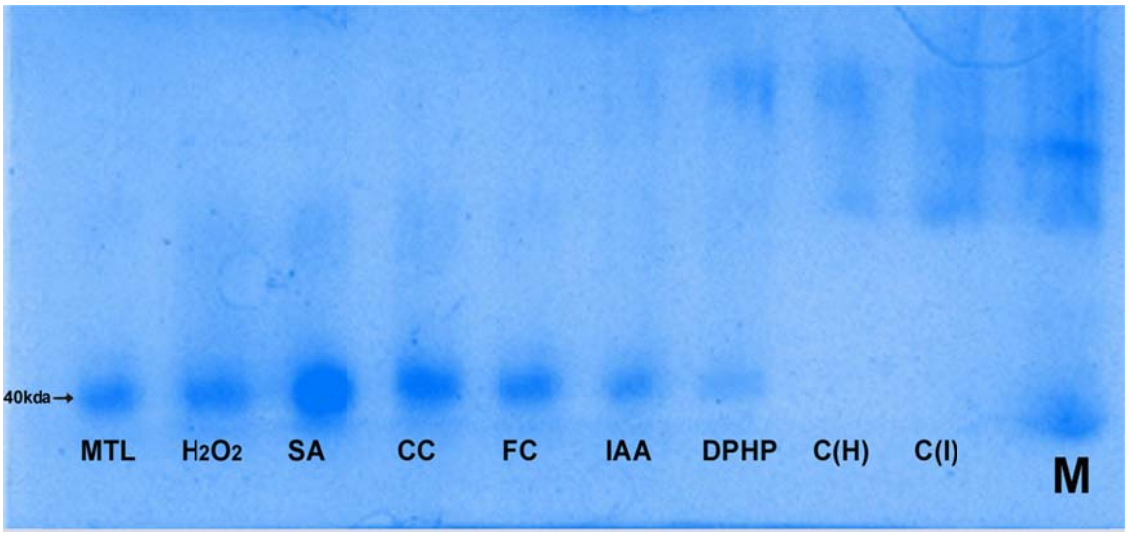

Figure 1. Banding pattern of protein on gel through SDS-PAGE (MTL- metalaxyl, H_2O_2- hydrgen peroxide, SA- salicylic acid, CC- calcium chloride, FC- ferric chloride, IAA- indole acetic acid, DPHP- di potassium hydrogen phosphate, C(H)- control healthy, C(I)- control infected and M- marker).

salicylic acid treated mustard plants, the phenol content had the maximum increase. Similarly, the increment of soluble protein in these plants was recorded as the highest. The accumulation of 40 KDa protein which seems to be associated with the disease resistance in the salicylic acid treatment was in substantial amount. Correspondingly, the disease severity in these plants was the minimum. Thus it may be presumed that inducers treated plants on being challenged by the pathogen synthesized greater amount of phenol and soluble proteins. Simultaneously, a new type of defense related protein (40 KDa) is produced and accumulated in considerable amount. The biochemical changes brought about by the abiotic inducers seem to be responsible for lower disease index. On the contrary, in the untreated plants and non-inoculated plants, the phenol and soluble protein content were significantly low and the 40 KDa protein was totally absent. However, minimum amount of phenol and soluble protein were estimated in the elicitor untreated but inoculated plants. The defense response and the disease severity in other treatments are intermediary of these two extremes.

Phenols are well known antifungal, antibacterial and antiviral compounds and are involved in disease resistance in many ways like hypersensitive cell death or lignifications of cell wall (Kumar, 2008; Kumawat et al., 2008; Nicholson and Hammerschmidt, 1992). Yadav et al. (2012) used salicylic acid as an inducer on mango and observed that phenolic compound, mangiferin content increased substantially. Mangiferin prevented the ingress of pathogen deep in the cells and finally killed the pathogen in the colonized host tissues. They also observed that salicylic acid treatment increased β-1,3 glucanase activity. β-1,3 Glucanase belongs to the group of PR- proteins and exhibits antifungal activity. Resistance to Alternaria blight in mustard has been found to be associated with leaf enzymes associated with the phenolic pathway (Singh et al., 1999). Earlier systemic acquired resistance (SAR) has been reported by inoculation with avirulent race of *A. brassicae* (Vishwanath et al., 1999).

Based on the present studies, it may be concluded that inducers treated plants on being challenged by the pathogen, synthesized greater amount of phenol and soluble proteins which significantly enhance disease resistance. The test compounds play the role of defense system elicitor and suggest that these applications hold a promise in substituting fungicidal control.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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