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Effect of pre- and post-inoculation application of amino acids on induction of resistance, peroxidase activity against *Albugo candida* (Pers) Kuntze in Indian mustard [*Brassica juncea* (L.) Czern & Coss.]

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Abstract

In the present investigation, the mustard genotype EC-399301 (RESJ-177) (white rust- susceptible at cotyledonary leaf stage but resistant at the true leaf stage), when treated with the amino acids at the cotyledonary leaf stage following inoculation application of *A. candida* showed no development symptoms in contrast to profuse development of white rust (WR) pustules on cotyledons of similarly inoculated check plant of the same genotype. Thus, revealed induction of host resistance of otherwise susceptible cotyledonary stage of EC-399301. No development of symptom due to the amino acids was characterized by expression of hypersensitive-like necrotic lesions on the cotyledons in L-proline (@1000 ppm), L-Glutamic acid (@1000 ppm), L-Cystine Hydrochocnloride (@1500 ppm) treatments. The significant high peroxidase activity was recorded in treatment L-Cystine at 2000 ppm (0.073 unit/mg) in comparison to check (0.04 unit/mg). Subsequent post inoculation application of *A. candida, n*o symptom development due to the amino acids was characterized by expression of hypersensitive-like necrotic regions on the cotyledons in all treatments of amino acids @ 2000 ppm except L-Tyrosine. The peroxidase activity was recorded in the range of 0.013-0.367 unit/mg in comparison to check (0.049 unit/mg). In both the cases of inoculation application of the above amino acids, hypersensitive response of the treated cotyledons was associated with concomitant increase in the peroxidase activity. The results, thus, revealed induction of host resistance of otherwise susceptible cotyledonary stage of EC-399301.

Key words: *Peroxidase, Albugo candida, amino acids, host resistance.*

Introduction

White rust (WR), more appropriately called as white blister or staghead disease appears in different proportion on rapeseed-mustard crop in several localities throughout the world (Kolte 1985a; b). White rust [A*lbugo candida* (Pers) Kuntze] is a serious disease causing heavy yield losses in mustard [*Brassica juncea* (L.) Czern & Coss.] (Bisht *et al*., 1994; Kolte, 1985 a; b). In recent years, a new group of chemicals that activate host defense mechanism and protect the plant against pathogens has been developed to manage crop diseases. These chemicals are called "plant defense activators" or "plant activators" (Romero *et al*., 2001). Salicylic acid mimic compound (acibenzolar-s-methyl, Bion), phosphorous salts (Foli-R-Fos 400, Nutri-Phite-P) and micronutrient potassium salts (Canon, Phytogard and Nutrol) have been developed as commercial plant activators (Graham and Leite, 2004; Becot *et al*., 2000; Macmillan *et al*., 2000; Pajot *et al*., 2001).

Some biological plant defense inducers such as *Trichoderma, Pseudomonas, Bacillus, Serratia*, non-pathogenic strains of *Fusarium* and yeast have been developed as commercial product to manage various diseases (Droby *et al.*, 2002; Benhamon and Garand, 2001; Varhagen *et al*., 2004; Howell *et al*., 2000).

Host resistance can be induced by the application of non-pathogenic microorganisms (Vishwanath *et al*., 1999; Singh *et al*., 1999) and certain chemicals such as salicylic acid (Spletzer and Enyedi, 1999)

and β – amino butyric acid (Cohen *et al*.; 1994; Kaur and Kolte, 2001). Plant adopts a variety of biochemical defense towards microbial attack. Various metabolic changes in plant tissues induced by biotic and abiotic inducers have been reported by various research worker (Benhamou and Belanger, 1998; Gorlach *et al*., 1996; Reuveni *et al*., 1997; Sticher *et al*., 1997; Singh *et al*., 1999; and Howell *et al*., 2000). It is well established that the resistance can be induced in plants by biotic as well as abiotic agents (Kessman *et al*., 1994; Sticher *et al*., 1997; Kaur and Kolte, 2001). However, genes in the susceptible plants can be activated to show resistance by inoculation with biotic as well as abiotic inducers (Vishwanath *et al*., 1999). Therefore, the exploitation of possibility of utilizing induced host resistance as a realistic alternative to classical fungicides in disease management needs to be explored.

In this context, the present investigation was carried out to study the mechanism of induction of host resistance due to pre- and post-inoculation application of amino acids against white rust true leaf- resistant genotype EC-399301 (RESJ-177) but susceptible at the cotyledonary leaf stage (Mishra *et. al.,* 2009).

Materials and Methods

In the present study, laboratory-cum-glasshouse experiments were conducted on white rust true leaf -resistant mustard genotype EC-399301 (RESJ-177) but susceptible at cotyledonary leaf stage at GBPUA&T, Pantnagar. The healthy seeds maintained through selfing were obtained from Oilseeds Pathology Laboratory, GBPUA&T., Pantnagar, which were earlier obtained through NBPGR under the Indo-UK collaboration Oilseeds (Kolte *et. al.,* 2006). All chemicals and reagents were of analytical and guarantee grade. Chemicals used in the study of Peroxidase assay were obtained from Sigma Chemical Company, USA.

Induction of resistance using cotyledons : The seeds were sown in plastic pots and arranged in propagator trays (41 cm x 30 cm x 7 cm) in three rows. Two to three seedlings were maintained per pot of the propagator trays. Seven-day-old seedlings with expanded cotyledons were used for inoculation.

Humid chambers : The humid chambers were prepared by putting the iron frame of 1.5×0.6×0.7m size over the cement pit of 1.45×0.45×0.45m size containing water and covered by plastic sheet. This helps in providing high humidity for infection. Four sets of such humid chambers were prepared maintaining the inoculating treatments in isolation treatments to avoid any drift. Another type of humid chamber was prepared by propagators trays of size 41x30x7cm containing water and covered by plastic sheet.

Induction of host resistance using amino acids

Preparation of A. candida inoculum : WR

Table 1 : Experimental treatments involving ten different acids and their concentrations

Amino acids	Treatment symbols	Concentration (ppm)
L-Proline	L-Proline	500, 1000, 1500, 2000
L-Lysine Monohydrochloride	L-Lysine Monohydrochloride	500, 1000, 1500, 2000
L-Tyrosine	L-Tyrosine	500, 1000, 1500, 2000
L-Leucine	L-Leucine	500, 1000, 1500, 2000
L-Cystine	L-Cystine	500, 1000, 1500, 2000
L-Arginine Monohydrochloride	L-Arginine Monohydrochloride	500, 1000, 1500, 2000
L-glutamic acid	L-glutamic acid	500, 1000, 1500, 2000
Glycine	Glycine	500, 1000, 1500, 2000
L-Cysteine Hydrochloride	L-Cysteine Hydrochloride	500, 1000, 1500, 2000
L-Histidine Monohydrochloride	L-Histidine Monohydrochloride	500, 1000, 1500, 2000
Unsprayed	Check	Distilled water only

infected leaves were collected from field grown WR-susceptible mustard cultivar "varuna" at the Crop Research Centre (CRC), GBPUA&T, Pantnagar and further maintained as generation of single WR pustule on the same cultivar on pot-grown mustard plants under glasshouse conditions. Zoosporangial powder was obtained by scrapping the surface of pustules with sterilized blade. Sporangia of *A. candida*, i.e. about 200 mg sporangial powder was added to 100 ml of double glass distilled water in 200 ml of flask. The flask was then covered with parafilm and shaken vigorously to obtain uniform suspension of sporangia in water. The culture was then incubated for about 4 hr. at 15^0C to obtain germination of sporangia so as to obtain zoospores suspension, which in the true sense served as the inoculum for inoculation of the cotyledons. The zoospore concentration was approximately adjusted to 10^5 zoospores per ml. Amino acids were used as given in the Table 1.

Induction of resistance using cotyledons :

1. Pre-inoculation of amino acids: Inocula of *A. candida* were prepared as described with ten different amino acids at 500, 1000, 1500 and 2000 ppm concentrations and were challenge inoculated after 24 hrs keeping suitable control as per details given in Table 2.

Table 2. Effect of pre-inoculation application of amino acids at different concentrations on reaction of mustard genotype EC-399301 at cotyledonary leaf stage against WR under glasshouse conditions

Treatment	Disease index (%) (15 DAI)* Concentration (%)				
	500	1000	1500	2000	Mean
L-Proline	1.8(7.6)	0.0(0.6)	1.8(6.6)	1.8(4.9)	1.4(4.8)
L-Lysine Monohydrocholoride	4.0(11.3)	7.7(15.3)	1.5(6.9)	0.3(2.2)	3.4(8.9)
L-Tyrosine	4.9(10.6)	3.1(8.3)	4.6(10.3)	1.2(4.1)	3. 5(8.3)
L-Leusine	0.3(2.2)	2.2(8.2)	8.6(15.1)	3.7(10.7)	3.7(9.1)
L-Cystine	0.3(2.2)	3.1(8.4)	1.8(4.9)	0.3(2.2)	1.4(4.4)
L-Arginine Monohydrochloride	2.1(6. 6)	4.3(11.9)	2.5(7.3)	0.3(2.2)	2.3(5. 7)
L-Glutamic acid	2.2(8.2)	0.0(0.6)	2.5(7.3)	1.8(6.6)	1.6(6.6)
Glycine	1.8(6.6)	2.8(9.5)	1.2(4.1)	0.6(3.9)	1.6(3.9)
L-Cystine Hydrochroride	1.8(6.6)	1.5(5.7)	0.0(0.6)	1.9(6.5)	1.3(6.5)
L-Histidine Monohydrochloride	1.2(5.4)	2.5(8.6)	3.1(9.8)	2.5(8.9)	2.1(8.2)
Check	39.1(38.7)	39.1(38.7)	39.1(38.7)	39.1(38.7)	39.1(38.7)
Mean	5.4(7.4)	6.0(8.6)	6.1(8.0)	4.9(6.5)	5.6(7.5)
C.D. at 5%					
Treatment					3.9
Concentration					2.4
Interaction					7.9

*DAI = Days after inoculation Figures in parenthesis are angular transformed values

2. Post-inoculation application of amino acids: In another experiment, cotyledons were first inoculated with *A. candida* and after 24 hrs, treatments of amino acids were given keeping suitable control (Table 4).

In each case of the two methods of inoculation, effect of amino acid with the same concentrations was tested by placing 2 drops (10 ml) with the help of Eppendorf pipette on adaxial surface of each cotyledon and the same leaf surface was used for inoculation of *A. candida*. Propogator trays were

Table 3: Effect of pre-inoculation application of amino acids on peroxidase activity against *Albugo candida* on mustard genotype EC-399301 using cotyledonary leaves

Treatment	Disease index (%)	Peroxidase activity (unit/mg)
T_5 L-Cys	0.3(2.2)	0.073
Check	39.1(38.7)	0.049
P=0.05	3.6	0.13

Figures in parenthesis are angular transformed values
Enzyme activity in leaves from some of the promising treatment /(s) were estimated

Table 4. Effect of post-inoculation application of amino acids at different concentrations on reaction of mustard genotype 'EC-399301' at cotyledonary leaf stage against WR under glasshouse conditions

Amino acid treatment (Aa treatment)	Disease index (%) (15 DAI)* Aa concentration (ppm)				
	500	1000	1500	2000	Mean
L-Proline	2.8(9.6)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.7(1.8)
L-Lysine Monohydrochloride	2.8(7.7)	0.0(0.0)	0.0(0.0)	0.0(0.0)	1.8(5.9)
L-Tyrosine	0.0(0.0)	0.0(0.0)	2.8(7.7)	3.7(10.9)	1.6(4.7)
L-Leusine	0.0(0.0)	2.8(9.6)	0.0(0.0)	0.0(0.0)	0.7(2.4)
L-Cystine	2.8(7.7)	2.8(7.7)	0.0(0.0)	0.0(0.0)	1.4(3.9)
L-Arginine Monohydrochloride	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)
L-Gluamic acid	0.0(0.0)	0.00(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)
Glycine	0.0(0.0)	2.8(9.6)	2.8(9.6)	0.0(0.0)	1.4(4.8)
L-Cysteine Hydrochloride	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)
L-Histidine Monohydrochloride	0.0(0.0)	2.8(3.2)	0.0(0.0)	0.0(0.0)	0.7(0.8)
Check	38.6(38.9)	38.6(38.9)	38.6(38.9)	38.6(38.9)	38.6(38.9)
Mean	4.3(5.0)	4.5(5.4)	4.0(4.5)	3.9(4.2)	4.4(5.0)
P=0.05					
Treatment					2.1
Concentration					1.3
Interaction					4.3

*DAI = Days after inoculation
Figures in parenthesis are angular transformed values

covered with polythene to obtain 90 to 100 per cent relative humidity for 72 h r.

Observation on WR severity:

Observations on WR severity on cotyledonary leaves were recorded 15 days after inoculation by using an interaction phenotype (IP) rating scale of 0 - 9e given by Williams (1985) where, IP of 0-1(no or traces of WR infection) was rated as resistant hypersensitive reactions and IP of 7-9 (profuse WR symptoms on lower surface of cotyledons) was rated as susceptible reaction. The disease (WR) index on cotyledons was calculated as: (Sum of all numerical rating x 100)/ (maximum grade x number of cotyledons examined).

Leaves were collected randomly from 2000 ppm concentration and check treatments. Immediately

after sampling, the leaf materials were wrapped in aluminium foil, weighed and stored in 1 gm packs at –80^{0}C. Then extracted supernatant was used for peroxidase assays.

Peroxidase enzyme assay:

Extraction of crude enzyme : One gram of fresh leaf tissue was macerated in 3 ml of 0.1 M extraction buffer (pH 7.0) in a prechilled mortar with pestle at 4^{0}C for 20 minutes. The supernatant was aspirated with the help of micropipette and was kept on ice till the assay was carried out. This mixture was used as crude enzyme for estimation of peroxidase. Peroxidase activity was measured at 510 nm.

Reagents : 0.2 M potassium phosphate buffer (pH 7.0) 0.0017 M H_2O_2 prepared by dilution of 1 ml of 30 per cent H_2O_2 to 100 ml with reagent grade water and further diluted 1 ml of this solution to 5 ml with 0.2 M phosphate buffer (pH 7.0) prepared afresh. 0.0025 M amino antipyrine with 0.17 M phenol was prepared by dissolving 810 mg phenol in 40 ml reagent grade water and 25 mg 4 amino antipyrine was added and diluted to final volume of 50 ml with grade water.

Enzyme assay : One mg sample in one ml of reagent grade water was dissolved immediately prior to use and diluted further to obtain a rate of 0.02 to 0.04 DA per minute. The spectrophotometer was adjusted to 510 nm and 25 ml pipetted into each cuvette as first phenol/amino antipyrine solution (1.4 ml) and second 0.0017 M H_2O_2 (1.5 ml). Reaction mixture was incubated in spectrophotometer at 25^{0}C for 3 to 4 minutes to achieve temperature equilibrium and blank reaction establishment. One ml of dilute enzyme was added and the increase in A 510 for 4 to 5 minutes was recorded. Calculated DA 510 per minute from linear portion of curve. Peroxidase activity (units/ mg) was calculated by divided 6.58xmg of enzyme per ml reaction mixture with ÄA510 per minute.

Results and Discussion

Effect of pre-inoculation application of amino acids on disease intensity of white rust on cotyledons: After fifteen days of pre-inoculation application of amino acid, highly significant difference was found among treatment concentration and interaction (Table 2). It was observed that highly significant minimum disease index was observed at cotyledonary leaf stage when it was pre-inoculated with L-Cystine Hydrochroride (1.3%), which was at par with L-Proline (1.4%), L-Cystine (1.4%), Glycine (1.6%) and L-Glutamic acid (1.6%). Slight increase in disease index was observed in L-Histidine Monohydrochloride (2.1%) which was at par with L-Arginine Monohydrochloride (2.3%). In treatment L-Lysine Monohydrocholoride (3.4%) disease index was observed at par with L-Tyrosine (3.5%) and L-Leusine (3.7%) in comparison to check (39.1%).

Effect of post-inoculation application of amino acid on disease intensity of white rust on cotyledons: After fifteen days of post-inoculation application of amino acid, highly significant difference was found among treatment concentration and interaction in genotype EC-399301 at cotyledonary leaf stage (Table 4). It was observed that L-Cystine Hydrochroride, L-Glutamic acid d and L-Arginine Monohydrochloride recorded (0.0%) disease index at par with L-Proline (0.7%) and L-Histidine Monohydrochloride (0.7%). The disease index in Glycine was (1.4%), L-Tyrosine (1.6%) and L-Lysine Monohydrocholoride (1.8%).

The mustard genotype EC-399301 (RESJ-177) (WR susceptible at cotyledonary leaf stage but resistant at true leaf stage), when treated with the amino acids at the cotyledonary leaf stage following post-inoculation application of *A. candida* showed no development of symptoms in contrast to profuse development of WR pustules on cotyledons of similarly inoculated check plant of the same genotype (Table 4).

The results thus revealed that the amino acids can be better used to induce resistance in the host by following pre and post-inoculation method. This also indirectly reveals the curative effect of these amino acids against WR infection of mustard. In the present studies, the amino acids as tested for their *in vitro* effect on *A. candida* indicated the tendency of reduction of sprorangial germination of *A. candida*. However, the result on inhibition on the fungal growth was non-significant.

Effect of amino acids on Peroxidase activity:

In the present investigation, the results thus revealed induction of host resistance of otherwise susceptible cotyledonary stage of EC-399301 (RESJ-177). The mustard genotype EC-399301 (RESJ-177) (WR susceptible at cotyledonary leaf stage but resistant at true leaf stage), when treated with the amino acids at the cotyledonary leaf stage following pre-inoculation application of *A. candida* showed no development of symptoms in contrast to profuse development of WR pustules on cotyledons of similarly inoculated check plant of the same genotype. No symptom development due to the amino acids was characterized by expression of hypersensitive-like necrotic regions on the cotyledons in L-Proline (@1000 ppm), L-Glutamic acid (@1000 ppm), L-Cystine Hydrochloride (@1500 ppm) (Table 2).

Pre-inoculation application of L-Cystine at 2000 ppm, on cotyledonary leaves showed highly significant differences over check in peroxidase activity (Table 3). The significant maximum peroxidase activity was recorded in this treatment i.e. L-Cystine at 2000 ppm (0.073 unit/mg) in comparison to check (0.049 unit/mg), which resulted in expression of lowest disease index (0.3%) due to L-Cystine in comparison to check (39.1%).

The results thus revealed induction of host resistance of otherwise susceptible cotyledonary stage of EC-399301 (RESJ-177). No symptom development due to the amino acids was characterized by expression of hypersensitive-like necrotic regions on the cotyledons in L-Arginine Monohydrochloride, L-Glutamic acid and L-Cystine Hydrochloride (@500, 1000, 1500, 2000 ppm) when treated with the amino acids at the cotyledonary leaf stage following post inoculation application of *A. candida* (Table 4).

The post-inoculation application of different amino acids on cotyledonary leaves of mustard genotype EC 399301 indicated maximum peroxidase activity (0.367 unit/mg) due to Glycine, (0.303 unit/mg), L-Arginine Monohydrochloride and (0.086 unit/mg) and L-Lysine Monohydrocholoride in comparison to check.. All amino acid treatments following post inoculation showed no development of WR symptoms as against 38.6% disease index of 38.6% in the case of check (Table 5).

The induction of host resistance due to pre and post-inoculation application amino acids was found to be associated with enhanced activity of peroxidase enzymes. The role of salicylic acid in the induction of host resistance has also been reported by several

Table 5. Effect of post-inoculation application of amino acids @ 2000 ppm on peroxidase activity against *Albugo candida* on mustard genotype EC-399301 using cotyledonary leaves

Treatment	Disease index (%)	Peroxidase activity (unit/mg)
L-Proline	0.0(0.0)	0.033
L-Lysine Monohydrochloride	0.0(0.0)	0.086
L-Tyrosine	3.7(10.9)	0.066
L-Leucine	0.0(0.0)	0.022
L-Cystine	0.0(0.0)	0.033
L-Arginine Monohydrochloride	0.0(0.0)	0.303
L-Glutamic acid	0.0(0.0)	0.055
Glycine	0.0(0.0)	0.367
L-Cysteine Hydrochloride	0.0(0.0)	0.019
L-Histidine Monohydrochloride	0.0(0.0)	0.013
Check	38.6(38.9)	0.049
P=0.05	1.2	0.12

Figures in parenthesis are angular transformed values
Enzyme activity in leaves from 2000 ppm conc. in acids treatment (s) were estimated

other workers in different host-pathogen systems (Yalpani *et al*., 1991; Sticher *et al*., 1997; Harman, 2000; Rohilla *et al*., 2001). Enhanced activity of peroxidase leads to the production and accumulation of hydroxyproline rich glycoprotein (HRCP) into the cell wall (Bradley *et al*., 1992). Peroxidases are also involved in lignin polymerization. The enzyme has multifaceted role in cell wall metabolism as well as in defense (Reuveni *et al*., 1997; Singh *et al*., 1999).

The enzyme is involved in the synthesis of lignin, suberin and catalyze the oxidation of many mono and diphenol aromatic amines were highly toxic in the presence of hydrogen peroxide. The enzyme is also involved in cross-linking of polyphenol to the HRPGs molecules to strengthen the cell wall (Hammerschmidt and Kuc, 1992). Systemic protection of *B. juncea* against the compatible isolate by treatment with incompatible isolate of *A. candida* is host-mediated with increased peroxidase and PAL- activity (Singh *et al*., 1999).

This information on the effectiveness of amino acids on reaction of mustard to WR infection will be useful in designing and understanding the mechanism of resistance in future studies. WR- true leaf resistant mustard genotype EC-399301 but susceptible at the cotyledonary leaf stage showed differences in response to application of amino acids indicates the interaction between the host genotype and pathogen.

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