Plant Archives Vol. 20, Special Issue (AIAAS-2020), 2020 pp. 302-306 *ISSN 0972- 5210*

EFFECT OF INOCULATION WITH *A. CANDIDA* SPORE SUSPENSION ON THE LEAF CELL WALL NON-CELLULOSIC POLYSACCHARIDES (TFAA HYDROLYSABLE) OF MUSTARD VARIETIES

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Abstract

Mustard is a major rabi seasonal crop, which accounts for more than 80% of the total cultivated area in the Indian subcontinent. This is having major significance for farmers, the edible oil industry, and other linked enterprises resulting in India fifth-largest oil-producing country and the biggest importer of vegetable oils. Different forms of Brassica are grown in India. *Albugo candida* an obligate parasite responsible for White Rust disease-causing extensive distortion "stag heads" phase accounts for major yield loss. To study the differential response in cell wall polysaccharide and hydroxyproline rich glycoproteins in resistant and susceptible cultivars of mustard, cell walls were isolated from one-month-old leaves of uninoculated and inoculated with a zoo sporangial suspension of *Albugo candida*. Changes in leaf cell wall constituents like non-cellulosic polysaccharides, cellulosic polysaccharides, polyuronides content and hydroxyproline (HRGPs) content after inoculation in resistant and susceptible cultivars of mustard were studied.

***Key words*:** *Brassica juncea*, Rabi crop, *Albugo candida*, Non cellulosic & cellulosic Polysaccharides, Polyuronides and Hydroxy proline-rich glycoproteins (HRGPs).

Introduction

The edible oil crisis at national level is really alarming and deserves utmost priority. Indian mustard (*B. juncea* L.) is a major *rabi* oilseed crop of the Indian subcontinent occupies more than 80% of the total rapeseed-mustard cultivated area. The enhancement in production and productivity of the crop assumes significance, not only for farmer's viewpoint but also to the edible oil industry and other vertical and horizontally linked enterprises and stakeholders. Indian vegetable oil economy is the fourth largest in the world after USA, China and Brazil. It accounts for 7.4% of worlds oilseed output; 6.1% of oil meal production; 3.9% oil meal export; 5.8% vegetable oil production; 11.2% oil import and 9.3% of the worlds edible oil consumption. In India, oilseeds contribute nearly 3.0% to gross national products and 10% to the value of all agricultural products respectively. Mustard is *Rabi* seasonal crop especially winter season crop are sown in October-November and harvesting time is February-March (Raliya *et al.*, 2018).

Total area under Mustard crop in India for the year 2016-17 was 66.52 lakh hectares as per the Government's estimates, estimated total production of Mustard was 71.09 lakh tones. In this year average yield was 1069 Kg per hectare. About 14.0 million farmers are involved in oilseed cultivation and 1.0 million in processing. Despite being the fifth largest oilseed producing country in the world, India is also one of the biggest importers of vegetable oils. There is a Spurt in the vegetable oil consumption in recent years, both for edible purposes as well as for industrial uses. The per capita consumption of oil which was only 2.5 Kg in early 50's has reached to about 14.0 Kg in recent years.

Out of seven edible oilseed crops cultivated in India, rapeseed mustard occupies second position in area and production next to groundnut sharing 27.80% in the India oilseed economy and countries 28.60% in the total oilseeds production (Shekhawat *et al.*, 2012). Rajasthan has exceeded all other states in the country in the production of this important oil crop. Different forms of *Brassica* are grown in India. Brown sarson [*Brassica Campestris* (Linn.) var. Dichotoma Watt] is getting out of cultivation due to its high susceptibility to aphids, and diseases like Alternaria blight, white rust and downy mildew. While yellow sarson [*Brassica campestris* (Linn.) var. Sarson Prain] is not potentially cultivated because of high inputs required limiting it to affluent farmers only.

Albugo candida (Pers. Ex. Lev.) Kuntze. (*A. cruciferarum* S.F. Gray), a member of the family Albuginaceae in the order Albugonales of class Peronosporomycetes is an obligate parasite responsible for causing white rust (WR) disease of many cruciferous crops (Saharan and Verma, 1992). Local infection produces white to cream colored pustules on leaves, stems and pods, while

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general or flower bud infection (Verma and Petrie, 1980) causes extensive distortion, hypertrophy, hyperplasia and sterility of inflorescences generally called "stag heads". The stag head phase (SP) accounts for most of the yield loss attributed to this disease.

A number of reports indicate that the respiration rates of tissues infected by members of the *Albuginaceae* also rise dramatically (Black *et al*., 1968, Williams and Pound, 1964). Long and Cooke (1974) suggested that host-fungus movement of carbohydrates in *Albugo-Senecio squalidus* system is maintained by hydrolysis of host sucrose and uptake of hexoses, followed by accumulation of trehalose within the mycelium and spores. Trehalose was synthesized within pustules by the fungus but no acyclic polyols were found. Accumulation of hexoses around pustules together with increased hydrolysis of exogenous sucrose by pustular material indicated increased invertase activity within infected tissues. Accumulation of dark fixed carbon compounds in WR pustules of *Senecio squalidus* infected with *A. tragopogonis* has been reported (Thomton and Cooke, 1970). Quantitative imaging of chlorophyll fluorescence revealed that the rate of photosynthesis declined progressively in the invaded regions of the leaf. Images of non-photochemical fluorescence quenching (NPQ) suggested that the capacity of the Calvin cycle had been reduced in infected regions, and that there was a complex metabolic heterogeneity within the infected leaf. *Albugo candida* also caused localized changes in the carbohydrate metabolism of the leaf; soluble carbohydrates accumulated in the infected region whereas the amount of starch declined. There was an increase in the activity of invertases which was confined to regions of the leaf invaded by the fungal mycelium. The increase in apoplectic interties activity was of host origin, as mRNA levels of the ATB FRUCT1 gene (measured by semi quantitative RT-PCR) increased 40-fold in the infected region. The increase in soluble invertase activity resulted from the appearance of a new isoform in the invaded region of the leaf. The resistant and moderately resistant cultivars contained higher amounts of chlorophyll, sugars and total phenols than the susceptible cultivar at all growth stages. However, total proteins and free amino acids were higher in the susceptible cultivar at all growth stages (Singh, 2000).

Materials and Methods

An experiment was conducted to study the changes in cell wall composition of mustard (*Brassica juncea*) leaves in relation to inoculation with (*Albugo candida*).

A. Seeds: Selfed seeds of two susceptible [T-59 (Varuna) and Pusa Bold] and two resistants' [PHR-1 and RW-8159] were obtained from oil seed scheme department of plant breeding Hisar.

B. Plant material: The seeds of both susceptible and resistant varieties were sown in different pots separately. One set is inoculated and another is uninoculated. Inoculation done with a spore suspension of *Albugo candida* at a known concentration of 104 zoospores per ml during evening hours when they were 30 days old. Three replications were taken for each treatment. Leaf samples were drawn from each of the treatments 60 hours after inoculation.

Cell wall preparation: Leaves of healthy plants (uninoculated) and from those challenged with *Albugo candida* were used for cell wall preparation. The required quantity of leaves of each variety was separately ground in liquid nitrogen. The cell contents were removed by suspending the powdered material in phosphate buffer [250 mM pH (7.0)] and centrifuged at 8,000 rpm for 5 minutes.

After 5 minutes the pellet was resuspended and the process repeated three to four times until the final supernatant was clear. The pellet was then finally washed twice with chloroform (CHC13):Methanol (1:1) and dried. The dried cell wall was stored in the freezer (-5°C) carefully.

Hydrolysis of Non-Cellulosic Cell wall polysaccharides (Nevins *et al*., 1967): Non-cellulosic polysaccharides were selectively hydrolyzed by 2 N trifloroacetic acid. Dried cell wall (10 mg) was introduced into clean dry test tube and 2 ml of 2 N trifluoroacetic acid was added. Tubes were sealed and heated at 121°C for one hour. Following hydrolysis, the tubes were opened. The residue was washed with 70 percent alcohol and centrifuged at 3,000 rpm for 10 minutes. The supernatants were collected and dried on water bath and dissolved in 2 ml distilled water and filtered. The sugar composition was analyzed by thin layer chromatography. The residue was preserved of the estimation of cellulosic polysaccharides. The reducing sugar content was estimated in the trifluoroacetic acid (TFAA) hydrolysate by the colorimetric ultra-micro assay method of Avigad (1975). This was taken as an index of the non-cellulosic polysaccharide content in the cell wall preparation.

Separation of Non-cellulosic sugars by thin layer chromatography (Ragazzi and Veronese, 1965) Procedure: Silica gel (35 gm) was suspended in 100 ml of M/15 phosphate buffer pH 8.0 and 0.25 mm thick layer was spread on 20 x 20 cm glass plates with a spreader. The plates were air dried followed by activation at 100°C for 15 minutes. 10µl of non-cellulosic polysaccharide hydrolysate was spotted on these plates, 3-4 cm above the bottom and developed in n-Butanol-acetone-water (4:5:1). The plates were air dried and sprayed with Ammonical silver nitrate (1.70 gm AgNO3 in 1 ml H2O + 1 ml ammonia solution in 35 ml methanol). The plates were kept at 100°C for one hour. The sugars were identified by comparing their Rf values with those of standard sugars.

Hydrolysis of Cellulose (Gross and Wang, 1984): The residue left after TFAA hydrolysis was washed with 1 ml of distilled water and 1 ml of 72 percent sulfuric acid was added. The samples were incubated at 30°C for 1 hour. The samples were diluted to a total volume of 25 ml with glass distilled water and autoclaved for 1 hour at 121°C. The reducing sugar content in the hydrolyzate was estimated by the method of Avigad, 1975 as described. This was taken as. An index of cellulosic polysaccharides.

Estimation of Uronic Acids (Modified Carbazole Method, Davidson, 1966):

Reagent A: Sodium tetra borate 0.025 M prepared in concentrated sulfuric acid.

Reagent B: Carbazole 0.125 percent in ethanol (store in cold).

Procedure: 5 mg cell wall was digested with 2 ml of 2 M sulfuric acid filtered with glass fiber filter (Whatman GF/A) and washed three times with 1 ml of distilled water 1 ml of the digest was treated with 5 ml of reagent A, the solution after thorough mixing was heated for 10 minutes in boiling water bath and cooled to room temperature. 0.2 ml of reagent B was added and mixture vortexed thoroughly. The resulting solution was heated for 15 minutes in a boiling water bath cooled and the absorbance was read at 530 nm with Hitachi-U-2000 spectrophotometer.

Results

To study the differential response in cell wall polysaccharide and hydroxyproline rich glycoproteins in resistant and susceptible cultivars of mustard, cell walls were isolated from one month old leaves of mustard varieties, susceptible (T-59 and Pusa Bold) or resistant (PHR-1 and RW-8159) challenged inoculated with zoosporangial suspension of *Albugo candida* and their composition determined.

A. Cell wall noncellulosic polysaccharides content of healthy and *A. candida* infected leaves: The leaf cell wall noncellulosic polysaccharides of healthy and Al2, 222 candida inoculated mustard plants are presented in Table 1. The cell wall non-cellulosic polysaccharides were significantly lower in leaves of inoculated plants as compared to that from healthy plants. Variation in the lowering of non-cellulosic polysaccharide content following inoculation was limited, ranging from 7.29 percent to 19.96 percent (Table 2). The percent decrease in susceptible varieties T-59 and Pusa bold was 7.29 percent and 12.13 percent while it was 19.90 percent and 19.96 percent in resistant varieties PHR-1 and RW-8159.

B. Cell wall cellulosic polysaccharides content of Healthy and *A. candida* infected leaves: The leaf cell wall cellulosic polysaccharides of healthy plants were significantly higher in the resistant varieties PHR-1 and RW-8159 compared to the susceptible varieties T-59 and Pusa Bold. Amongst the susceptible varieties, however the cell wall cellulosic polysaccharides were significantly higher in variety Pusa Bold as compared to T-59. The leaf cell wall cellulosic polysaccharides of healthy resistant plants did not significantly differ from one another.

Evidence presented in Table 3 suggests that the cellulosic polysaccharide content of cell wall decreased in susceptible cultivars, while it was significantly increased in resistant varieties following inoculation. Inoculation decreased the cellulosic polysaccharide content in susceptible varieties, T-59 and Pusa Bold by 7.62 percent and 9.49 percent respectively while in the resistant Varieties cellulosic polysaccharides increased by 18.24 percent in PHR-1 and 24.39 percent in RW-8159 (Table 4). Both the factors (variety and inoculation) and interaction variety x inoculation of cellulosic polysaccharide contents of cell walls were statistically significant (Table 3).

Discussion

The leaf cell wall non-cellulosic polysaccharides (Hemicelluloses) were also decreased in the resistant cultivars to a greater extent than the susceptible cultivars. The induction of phytoalexin capsidol in tobacco callus culture by an extracellular protein from Phytophthora parasitica with endoxylanase activity (Farmer *et al.*, 1987), induction of PR proteins in tobacco leaves by an endoxylanase from Trichoderma viride (Lotan and Flair, 1990) and ethylene synthesis in leaf discs of the same plant (Fuchs *et al.*, 1989) indicate that Xylan containing oligosaccharides may also be involved in signaling events during pathogenesis. Bucheli *et al*. (1990) have suggested that an endoxylanase and arabinosidase may work in concert reaction in rice plant in to produce hypersensitive response to the rice blast fungus Magnaport grisea (Bucheli *et al*., 1990). Some defense responses like the induction of PR proteins (β-glucanase and chitinase) have been shown to be elicited by xylanase (Lotan and Fluhr, 1990) as well as an Oligogalacturonide (Brockaert and Pcumans, 1988). Phenols, in particular have been reported to impart resistant whereas more protein lead to higher disease severity (Yadav *et al*., 1996; Pruthi *et al*., 2001, Pahuja and Sangwan, 2002) thus suggesting their role in disease resistance. It may well be speculated that oligosaccharides released by partial degradation of the host cell wall could function in concert to signal one or more defense responses in mustard leaf tissue leading to the resistance reaction in the resistant cultivars.

The cellulose content was found to be decreased in the leaf cell wall of mustard varieties susceptible to *A. candida* and increased in the leaf cell wall of resistant varieties following attempted infection. To the beat of our knowledge the increase in cellulose content by fungal inoculation is being reported for the first time. It is likely that the increased synthesis of cellulose may be required to provide the matrix essential for the deposition of HRGP and lignin. Enhancement of HRGP and cellulose may therefore be a part of the defense response in mustard contributing to varietal resistance.

The polyuronide contents of the mustard leaf cell wall were lower in inoculated compared to uninoculated plants of both susceptible and resistant cultivars. The magnitude of decrease however, was higher in the resistant (P1)0-1 and RW-8159) compared to susceptible (T-59 and Pusa Bold) cultivars. Gligogalacturonides are known to elicit defense responses like biosynthesis of lignin in castor beans (Bruce and West, 1989), β-glucanase and chitinase in tobacco (Broeckheart and Peumans, 1988), hypersensitive necrotic in *Vigna* (Cervone *et al.*, 1987) proteinase inhibitor synthesis in tomato leaves (Farmer *et al.*, 1991).

Pruthi *et al.* (2001) studied the relationship between disease severity and effect on glucosinolate content, the anti-nutritional Sulphur containing factor in oil meal. They reported an increase in glucosinolate content as the resistant plant were attacked by white rust pathogen no increase in glucosinolate content was observed susceptible plants of *B. juncea* cultivar were infected. This suggested that glucosinolate play role in triggering systemic acquired resistance in plants to an attack by white rust.

Conclusion

Mustard plants of resistant (PHR-1 and RW-8159) and susceptible (0-59 and Puss Bold) varieties were grown in pots.

Half of the one-month old plants of each variety were challenge inoculated with zoosporangial suspension of *Albugo candida*. Leaf samples from both uninoculated and inoculated plants were drawn after 60 hours of inoculation. Cell wall was isolated from leaf samples. Changes in leaf cell wall constituents like non-cellulosic polysaccharides, cellulosic polysaccharides, polyuronides content and hydroxyproline (HRGPs) content after inoculation in resistant and susceptible cultivars of mustard were studied. Higher amount of cellulose and lower amount of HRGP was observed in resistant varieties as compared to susceptible varieties. No significant differences in cell wall non-cellulosic polysaccharides and polyuronides contents were observed between resistant and susceptible varieties.

Cell wall non-cellulosic polysaccharide content in both resistant and susceptible cultivars was decreased after inoculation, however per cent decrease was higher in resistant varieties than in susceptible ones. The cell wall cellulosic content was increased in resistant varieties but decreased in susceptible varieties after inoculation. A higher percent decrease after inoculation was observed in cell wall polyuronide content of resistant as compared to susceptible varieties. Although the cell wall HRGP was higher in susceptible varieties, the increase in HRGP following *A. candida* inoculation was greater in the resistant cultivars. The greater lowering of leaf cell wall non-cellulosic polysaccharides and polyuronides of resistant plant. indicates that the rate at which oligogalacturonides and other oligosaccharins are released from the cell wall may be involved in the elicitation of the defense responses in mustard to *A. candida* and may well be the determinants of resistance.

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Table 1: Effect of inoculation with LI, *A. candida* spore suspension on the leaf cell wall non-cellulosic polysaccharides (TFAA hydrolysable) of mustard varieties differentially susceptible to it

Variety	Reaction to *A. candida*	Cell wall non-cellulosic to polysaccharides (%)		Mean
		Uninoculated	Inoculated	
T-59	R	53.0429	49.1767	51.1098
Pusa Bold	R	46.2077	40.6036	43.4057
	S	46.8225	37.5038	42.1631
RW – 8159	S	52.9437	42.3766	47.6602
Mean		**49.7542**	**42.4152**	**46.0847**
			C.D. 1%	**C.D. 5%**
Variety		0.644	2.66	1.93
Inoculation		0.455	1.88	1.36
Variety x Inoculation		0.910	3.76	2.73

S = Susceptible, R = Resistant

Table 2: Percent decrease in Leaf 'cell wall non-cellulosic polysaccharides of susceptible and resistant varieties of mustard following *A. candida* inoculation

Variety	Cell Wall non-cellulosic polysaccharides %		Difference	Percentage decrease
T-59	53.0439	49.1767	3.8662	7.29
Pusa Bold	46.2077	40.6036	5.6041	12.13
PHR-1	46.8225	37.5038	9.3187	19.90
RW-5159	52.94.37	42.3766	10.5671	19.96

Table 3: Effect of inoculation with *A. candida* spore suspension on leaf cell wall cellulosic polysaccharides (H_2 SO_4 the 24 hydrolysable) of mustard varieties differentially susceptible to it

Variety	Reaction to *A. candida*	Cell wall cellulosic to polysaccharides (%)		Mean
		Uninoculated	Inoculated	
T-59	R	27.3060	25.5290	26.5825
Pusa Bold	R	33.0260	29.8900	31.4580
PHR-1	S	36.7990	43.5120	40.1555
RW-8159	S	36.1620	44.9820	40.572
Mean		**33.4057**	**35.9783**	**34.6920**
		S.Em. ±	**C.D. 1%**	**C.D. 5%**
Variety		0.293	1.210	0.878
Inoculation		0.207	0.855	0.621
Variety x Inoculation		0.414	1.711	1.240

S = Susceptible, R = Resistant

Table 4: Percent decrease/increase in leaf cell wall cellulosic polysaccharides of susceptible and resistant varieties of mustard following *A. candida* inoculation

Variety	Cell Wall non-cellulosic polysaccharides %		Difference	Percentage Response
T-59	27.6360	25.5290	2.107	(-)
Pusa Bold	33.0260	29.8900	3.136	(-)
PHR-1	36.7990	43.5120	6.713	(+)
RW-5159	36.1620	44.9820	8.820	(+)