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Anti-oxidative enzyme changes associated with chickpea calli tolerant to *Ascochyta rabiei* culture filtrate

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Abstract

Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labrousse is a major biotic constraint in production of chickpea. In the present investigation, all chickpea genotypes [E100Y (m), Gaurav, Pb-7 and L550] induced 100% callus on standard medium with greenish colour and fragile structure. These calli were used for *in vitro* screening against pathogen, *A. rabiei* culture filtrate at 0, 5, 10, 15 and 20% concentrations. Survival rate of calli in all chickpea's genotypes were reduced significantly at higher concentration (15%) of culture filtrate. The culture filtrate concentration of 20 % was lethal for calli of all chickpea's genotypes. Hence, biochemical changes viz. total soluble proteins and activities of anti-oxidative enzymes (polyphenol oxidase, peroxidase and catalase) were estimated at 15% and below concentration of culture filtrate. Tolerant calli of resistant genotype, E100 Y (m) revealed significantly higher total soluble proteins (10.04 mg g^{-1} f.wt. of callus) and activity of anti-oxidative enzymes, polyphenol oxidase (9.0 unit absorbance change min^{-1} mg^{-1} protein) and peroxidase (19.09 unit absorbance change min^{-1} mg^{-1} protein) and lower catalase (18.65 µ moles of H_2O_2 utilized min^{-1} mg^{-1} protein) at higher (15%) concentration of culture filtrate followed by moderately resistant (Gaurav), and susceptible genotypes (Pb-7 and L550). Thus, higher polyphenol oxidase and peroxidase and lower catalase activity in chickpea's genotypes against culture filtrate of *A. rabiei* could be used as parameters for screening resistant genotypes to pathogen, *A. rabiei*.

Key words

Anti-oxidative enzymes, *Ascochyta rabiei*, Callus, Chickpea, Culture filtrate, Soluble proteins

Introduction

Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labrousse is the most destructive foliar disease of chickpea causing complete loss in grain yield under epidemic condition (Gaur and Singh, 1996; Pande *et al*., 2005). It is the most serious disease in the world, capturing 34 countries encompassing 5 continents Asia, Australia, North Africa, North America and South America (Kaiser *et al*., 2000). This disease devastates the crop in areas where cool, cloudy and humid weather (15^{0}C temperature and >150 mm rainfall) occurs during crop season (Nene, 1982). The pathogen secretes phytotoxic compounds solanapyrone A, B, C and cytochalasin D in liquid culture, impart their role in development of disease (Shahid *et al*., 2008). Mosconi *et al*. (1996) differentiated the resistant or susceptible plants or cell lines to *Ascochyta* blight using crude culture filtrate of *A. rabiei* and found that culture filtrate was toxic to chickpea cuttings, inhibits root elongation and causes ion leakage.

Genetic improvement of chickpea with respect to disease resistance using conventional breeding methods are being slow, because its narrow genetic bases of crop and presence of strong sexual incompatibility barriers with its wild relatives (Robertson *et al*., 1997; Mallikarjuna and Muehlbauer, 2011). Moreover, these methods are laborious, time consuming and need a large experimental area. Plant tissue culture techniques have been used extensively as alternative tool to accelerate the breeding process in many agricultural crops (Brown, 1995; Jain, 2001).

Tissue culture system facilitates screening of many genotypes within a short period and in a relatively smaller space by using crude or partially purified patho-toxins or culture filtrates (Song *et al*., 1994). This technique makes such cell cultures ideal for understanding the resistance responses and changes occurring at the cellular and sub-cellular levels, when infected with pathogenic organisms (Jayasanker, 2008). In the present investigation, an attempt was made to use the tissue culture technique for screening chickpea genotypes on callus stage with variable change in total soluble proteins and anti-oxidative enzymes viz., polyphenol oxidase, peroxidase and catalase in tolerant calli of chickpea genotypes on exposure to various concentrations of *A. rabiei* culture filtrate.

Materials and Methods

Isolation and purification of *Ascochyta rabiei*: *A. rabiei* was isolated from infected stems of chickpea, collected from experimental area of Plant Pathology, CCS HAU, Hisar on sterilized chickpea seed extract dextrose agar medium (20g chickpea seed extract + 20g dextrose + 20g agar $l^{-1}$of distilled water) at 20^{0}C. Isolated culture was purified by single spore isolation method (Punithalingam and Holliday, 1972).

Preparation of culture filtrate : Two fungal mycelial discs of 5 mm diameter were inoculated in 100 ml sterilized chickpea seed extract dextrose broth and incubated at 20^{0}C for 15 days. Culture filtrate was prepared by separating the fungal mycelium by passing broth culture through sterilized filter paper (Whatman No. 1) and then passed through 0.45 µm size Millipore filter under aseptic condition. The filtrate was used immediately for screening of chickpea calli.

Raising of chickpea's seedling and callus induction from hypocotyls explant : Seeds of four different chickpea genotypes viz. E100Y (m) (resistant), Gaurav (moderately resistant), PB-7 and L 550 (susceptible), to *Ascochyta* blight at field level were taken for callus induction. These seeds were collected from Pulses Division of C.C.S. H.A.U., Hisar. The AUDPC (Area Under Disease Progress Curve) values of E100Y (m) , Gaurav, PB-7 and L 550 genotypes were 20, 40, 65.5, and 70 respectively during 2008-2009 using disease rating scale (1-9) of Jan and Weise (1991). Seeds of these genotypes were soaked for 8-10 min in tap water containing few drops of teepol (a liquid detergent) followed by washing with tap water. Seeds were surface sterilized by immersing in 0.1% (W/V) mercuric chloride ($HgCl_2$) solution for 10 min(s), which were subsequently rinsed thoroughly four times with sterile double distilled water under aseptic conditions. Sterilized seeds were soaked in sterilized distilled water for 20 hrs and then inoculated on sucrose- agar medium (2% sucrose and 1% agar) for seedling raising and kept in the culture room at 25 ±1ºC under white fluorescent light for 16 hrs (1500 lux) and 8 hrs in dark condition for one week. Hypocotyls segment measuring 5-6 mm were excised from one-week-old-seedling under aseptic conditions and implanted on callus induction medium- MS basal (Murashige and Skoog, 1962) containing 2.5 mg l^{-1} IAA (indole-3-acetic acid) and 0.5 mg l^{-1} BAP (6-benzylaminopurine) for 20 days (Kumar *et al*., 2010). The pH of medium was adjusted to 5.8 using 1N NaOH or 1N HCl.

Effect of culture filtrate on calli of chickpea genotypes and biochemical estimation : Six calli of each genotype, E 100 Y (m), Gaurav, L 550, and Pb-7 was inoculated on each petriplates containing 25 ml callus induction medium with amendment of culture filtrate at 0, 5, 10, 15 and 20% concentrations. Each treatment was replicated four times and the experiment was designed in a complete randomized design (CRD). Cultured plates were kept in the culture room at 25 ±1ºC under 16 hrs white fluorescent light (1500 lux) and 8 hrs dark for 20 days (Naz *et al*., 2008). The calli which survived or were tolerant on treated medium were sub-cultured again one time on normal callus induction medium for acclimatization. Further, these calli were sub-cultured on their respective treated medium mentioned above for 20 days, and then biochemical estimation was carried out. For enzyme assay, 500 mg (fresh weight) of callus tissue from each category was hand homogenized in a pre-chilled mortar with 10 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.1 M L-cysteine and 0.01% ascorbic acid using acid washed sand as an abrasive. The homogenate was filtered through four layers of muslin cloth and then centrifuged at 10,000 X g for 20 min in a refrigerated centrifuge at 0-4^{0}C. The supernatant thus obtained was referred to as crude extract and stored in refrigerator for estimating enzyme activities. In each case, three separate extractions were made. The soluble proteins in the enzyme extract were precipitated by 20% trichloroacetic acid (TCA) and determined by the method of Lowry *et al.* (1951). The anti-oxidative enzyme activity of polyphenol oxidase (Taneja and Sachar, 1974), peroxidase (Shannon *et al*., 1966) and catalase (Sinha, 1972) were also determined using supernatant.

Results and Discussion

The calli were induced with 100% frequency on MS basal medium containing IAA (2.5 mg l^{-1}) and BAP (2.5 mg l^{-1}) from all four genotypes of chickpea [E100 Y (m), Gaurav, Pb-7 and L 550). The callusing was initiated from both cutting edges of hypocotyls explant. These were greenish and fragile. Calli induced from the individual genotype of chickpea showed brownish response to pathogen's culture filtrate as compared to control (without culture filtrate). The darkness in calli was enhanced positively with increase in the concentration of culture filtrate. 20% concentration of culture filtrate was lethal for calli of all four chickpea's genotypes. Few calli (8.33%) survived at 20% concentration of culture filtrate only in resistant genotypes E100 Y (m). The survival of calli was significantly higher (79.18%) at lower concentration (5%) as compared to higher concentrations (10 and 15%) in resistant genotype E100 Y (m) followed by moderately and susceptible genotypes (Table 1). Hence, sub-

lethal concentrations of culture filtrate at 15% and below were further used for *in vitro* screening of calli. The calli tolerant at these concentrations were used for biochemical studies. The levels of total soluble protein in tolerant calli of all four genotypes increased significantly ($p<0.01$) with increase in the concentration of culture filtrate (Table 2) as compared to control (without culture filtrate). The calli of resistant genotype [(E100 Y (m)] responded by accumulating higher total soluble proteins (10.04 mg g^{-1} fresh weight of callus) at 15% culture filtrate followed by moderately resistant (Gaurav), and susceptible genotypes (Pb-7 & L550). Activities of all the three anti-oxidative enzymes *viz.* polyphenol oxidase, peroxidase and catalase also increased significantly ($p<0.01$) in tolerant calli of all genotypes with an increase in concentrations of culture filtrate (Table 3). Polyphenol oxidase and peroxidase activities in calli of resistant genotype, E100 Y (m) were significantly higher 9.04 & 19.09 unit absorbance change min^{-1} mg^{-1} protein, respectively at 15% concentration of culture filtrate followed by moderately resistant (Gaurav) and susceptible genotypes (Pb-7 & L 550). Catalase activity was significantly higher (60.57 µM of H_2O_2 utilized min^{-1} mg^{-1} protein) in the susceptible genotype- L 550 followed by other susceptible genotype- Pb-7, moderately resistant- Gaurav, and resistant genotype- E100 Y (m). This is in contrast to polyphenol oxidase and peroxidase activities (Tables 3). Genotypes and culture filtrate interaction was significant ($p<0.01$) for anti-oxidative enzymes viz. polyphenol oxidase, peroxidase and catalase as well as for total soluble proteins.

Plants have complex systems to adapt to abiotic and biotic stresses. Among the response to stresses, increased production of reactive oxygen species (ROS) is one of them. ROS are formed due to incomplete reduction of oxygen. Oxidative burst involving production of ROS is a ubiquitous response of plants to pathogen attack (Torres *et al*., 2005). Over accumulation of ROS might enhance plant susceptibility (Govrin and Levine, 2000; Kariola *et al.,* 2005) or cause an uncontrolled defense with spreading cell death lesions that can kill the plant (Lorrain *et al*., 2003). ROS include a variety of short and long-lived molecules such as superoxide, hydroxyl radicals and hydrogen peroxide (Apel and Hirt, 2004). The level of ROS is maintained to optimum level by ROS-scavenging enzymes such as polyphenol oxidase, peroxidase and catalase.

The resistance responses in higher plants against microbial pathogens are result of constitutive and inducible defence mechanisms. After infection, the accumulation of phytoalexins (Mert-Turk, 2002) and synthesis of pathogenesis related proteins (Van Loon and Van Strien, 1999; Borad and Sriram, 2008) are prominent reactions in resistant varieties.

Peroxidase also produces free redicals and hydrogen peroxide which are toxic to many microorganisms (Pena and Kuc, 1992). Dhingra *et al*. (2004) reported that calli of *Brassica species* sub-cultured on medium having different concentrations of *A. brassicae* culture filtrate resulting in increased activities of

Table 1: Tolerant calli (%) of different chickpea genotypes exposed to various concentrations of *A. rabiei* culture filtrate

	R	MR	S		
Culture filtrate(%)	**E100Y (m)**	**Gaurav**	**Pb-7**	**L 550**	**Mean**
Control	100.00 (89.96) e	100.00(89.96) e	100.00(89.96) d	100.00(89.96) d	(89.96) e'
5	79.16(52.76) d	70.83(45.45) d	58.33(35.89) c	54.16(32.93) c	(41.76) d'
10	58.33(35.89) c	54.16(32.93) c	41.66(24.72) b	33.33(19.46) b	(28.25) c'
15	37.50(22.09) b	37.50(22.09) b	29.16(16.99) b	25.00(14.52) b	(18.93) b'
20	8.33(4.79) a	0.00(0.00) a	0.00(0.00) a	0.00(0.00) a	(1.20) a'
Mean	(41.10) b'	(38.08) b'	(33.51) a'	(31.38) a'	

R-Resistant , MR-Moderately resistant, S-Susceptible, Control-Without culture filtrateIndividual value indicate mean of 4 replications and value in parenthesis indicate angular transform; Different letter in each column indicate significant ($p<0.001$) by two factorial analysis of ANOVA

Table 2: Total soluble proteins (mg g^{-1} f.wt.) in calli of different chickpea genotypes exposed to various concentrations of *A. rabiei* culture filtrate

	R	MR	S		
Culture filtrate(%)	**E100Y (m)**	**Gaurav**	**Pb-7**	**L 550**	**Mean**
Control	4.24 a	3.88 a	3.35 a	3.12 a	3.65 a'
5	5.45 a	4.82 ab	4.30 ab	3.90 ab	4.62 b'
10	7.84 b	5.76 b	4.90 b	4.49 b	5.75 c'
15	10.04 c	7.46 c	6.51 c	5.13 bc	7.29 d'
Mean	6.89 d'	5.48 c'	4.77 b'	4.16 a'	

R-Resistant , MR-Moderately resistant, S-Susceptible, Control-Without culture filtrateIndividual value indicate mean of 3 replications and different letter in each column indicate significant ($p<0.001$) by two factorial analysis of ANOVA

Table 3: Polyphenol oxidase, peroxidase and catalase activities in tolerant calli of different chickpea genotypes exposed to various concentrations of *A. rabiei* culture filtrate

	Resistant	Mederately resistant		Susceptible	
Culture filtrate(%)	**E100Y (m)**	**Gaurav**	**Pb-7**	**L 550**	**Mean**
Polyphenol oxidase activity (unit absorbance change $min^{-1}mg^{1}$ protein)					
Control	3.81 a	3.10 a	2.34 a	1.87 a	2.78 a'
5	4.91 b	3.86 b	3.01 b	2.34 b	3.53 b'
10	7.06 c	5.61 c	3.43 c	2.69 c	4.70 c'
15	9.04 d	6.97 d	4.56 d	3.08 d	5.91 d'
Mean	6.21 d'	4.89 c'	3.34 b'	2.50 a'	
Peroxidase activity (unit absorbance change $min^{-1}mg^{1}$ protein)					
Control	7.21 a	5.82 a	3.02 a	2.63 a	4.67 a'
5	9.26 b	7.23 b	3.67 b	3.28 b	5.86 b'
10	14.11 c	9.79 c	4.89 c	4.17 c	8.24 c'
15	19.09 d	12.67 d	6.42 d	4.76 d	10.74 d'
Mean	12.42 d'	8.88 c'	4.50 b'	3.71 a'	
Catalase activity (µ moles of H_2O_2 utilized $min^{-1}mg^{1}$ protein)					
Control	10.26 a	11.64 a	23.45 a	25.98 a	17.83 a'
5	14.09 b	16.43 b	34.94 b	36.92 b	25.60 b'
10	16.32 b	19.28 c	45.57 c	49.08 c	32.56 c'
15	18.65 bc	25.84 d	53.63 d	60.57 d	39.67 d'
Mean	14.83 a'	18.30 b'	39.40 c'	43.14 d'	

R-Resistant , MR-Moderately resistant, S-Susceptible, Control-Without culture filtrateIndividual value indicate mean of 3 replications and different letter in each column indicate significant ($p<0.001$) by two factorial analysis of ANOVA

peroxidase and polyphenol oxidase in resistant cultivars than susceptible cultivars. But catalase activity was higher in calli of susceptible cultivars. The increases in total soluble proteins content were noticed in selected calli of *Brassica species*. Another supportive suggestion brought by Nawar and Kuti (2003) stated that an increase in peroxidase activity is considered as preliminary indicator for resistance of broad beans to chocolate spot disease. These compounds act as a barrier against pathogen invasion. Hassan *et al*. (2007) also revealed that increased peroxidase activity in faba bean in response to inducer molecule showed resistance against chocolate spot disease. Reuveni *et al.* (1992) reveled that higher activity of peroxidase in muskmelon showed resistant to pathogen *Pesudoperonospora cubensis*. Sindhu *et al.* (1998) reported that activity of peroxidase was increased in calli of both resistant and susceptible chickpea genotypes in responses to increased concentration of *Ascochyta rabiei* culture filtrate. Khan *et al*. (2001) reported that polyphenol oxidase and peroxidase enzyme activities increased in varying degree at different stages of infected leaf of sorghum with *Drechslera sorghicola*. Analysis of protein fractions showed significant increase with the progress of disease.

Peroxidase activity was also reported higher in selected calli of alfalfa against filtrate of *Fusarium* sp. as compared to non-selected calli (Hrubcova *et al*., 1992). When plants are exposed to different stressess either biotic or abiotic, results in shifting of their metabolism towards oxidative direction and plant mobilize the anti-oxidative defence mechanisms by several stress enzymes like peroxidase and catalase, in order to eliminate the effect of free radicals (Arora *et al*. 2002; Gill and Tuteja, 2010).

Jayasanker and Gray (2008) reported that when plant cells are subjected to such intensive *in vitro* selection, a number of defence genes are induced, however, only those cells whose defence mechanism is activated quickly in a sustained manner remain viable and survive recurrent *in vitro* selection. Plants regenerated from such cells exhibit certain native defense genes in a constitutive manner and thus are resistant as compared to their parental material. Jayasanker (2000) also revealed that plant cell and tissue culture has become an important tool in study of plant-pathogen interactions at the cellular and molecular levels. Plant cells react to certain biotic and abiotic stresses in a manner similar to that of intact plant. These techniques make such cell cultures ideal candidates for understanding the resistance responses and changes occurring at the cellular and sub cellular levels, when infected with pathogenic organisms.

The finding of the present investigation is critically important for *in vitro* screening of tolerant chickpea calli in responses to culture filtrate of pathogen, *A. rabiei* causing blight in

chickpea that showed considerable effect on survival and changes in oxidative enzymes viz. polyphenol oxidase, peroxidase and catalase.

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