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INVOLVEMENT OF ANTIOXIDANTS AND TOTAL PHENOLICS IN *GLYCINE MAX* L. RESISTANCE AND SUSCEPTIBILITY TO CHARCOAL ROT

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ABSTRACT

The study was conducted in potted soil to screen out soybean (*Glycine max* L. Merrill) genotypes against the effects of a destructive pathogen *Macrophomina phaseolina*. Soybean genotypes (AJMERI, PSC-60, RAWAL-I, NARC-II and NARC-I) were artificially inoculated with the pathogen kept in a completely randomized designed in triplicate for 45 days. Based on the disease severity, genotypes were categorized into four groups i.e., resistant (AJMERI), moderately resistant (PSC-60 and RAWAL-I), susceptible (NARC-II) and highly susceptible (NARC-I). The infection of pathogen led to many morphological and biochemical changes including reduction in growth attributes, up regulation in total phenolics and activities of the antioxidant enzymes (peroxidase, polyphenol oxidase and catalase) in susceptible groups but not in resistant groups. Therefore, the growth inhibition index was significantly increased in susceptible genotypes. The results of study suggested that the currently investigated physiological and biochemical attributes in infected plants could be used to assist the screening of resistant plants in soybean.

Keywords: *Macrophomina phaseolina*, pathogenicity, susceptible, resistant, antioxidant enzyme

INTRODUCTION

Soybean (*Glycine max* L. Merrill) is an important annual, herbaceous and leguminous crop of family Fabaceae (Hamel *et al.,* 1991), which is native to eastern Asia being cultivated from 11th century B.C. around the world (Hymowitz and Newell, 1981). It is an indeterminate strategic crop of 90-100 days, serves as a tremendous nutritive source of 40% digestible protein, 22% edible oil, 30% carbohydrates and 85% cholesterol free unsaturated fatty acids. It is rich in numerous minerals, vitamins and antioxidant enzymes as well (Yimit *et al.,* 2011). The United States, Brazil, Argentina, China, Asia and Canada are the leading producers of soybean. In Pakistan, its domestic production has hardly meet 18% of the total requirements. Due to the lack of supportive Govt. policies yield of 59 tons was recorded from 89 hectare, which is low (Anonymous, 2015). Besides abiotic constraints such as drought, heat, and cold etc., biotic stresses caused by pathogenic microbes are the major threats to soybean and responsible for 10-30% yield losses (Saleh *et al.,* 2011; Rahayu, 2014). Charcoal rot caused by seed and soil born fungus *Macrophomina phaseolina* (Tassi) Goid is a severe disease that plays crucial role in reducing yield of the crop (Vasebi *et al.,* 2013). *M. phaseolina* is polyphagous pathogen that can infect over 500 plant species in 100 families of monocots and dicots (Rayatpanah and Dalili, 2012). Over 67 hosts of *M. phaseolina* including wheat, rice, maize, cotton, cucurbits, okra and beans have been reported in Pakistan (Yang and Navi, 2005; Khan, 2007; Khan *et al.,* 2016). In soybean, *M. phaseolina* caused charcoal rot with significant losses (Sinclair and Gray, 1972; Bradley and Rio, 2003). Infected plants show typical symptoms at maturity. Vascular obstruction by mycelium and the production of toxins (phaseolinone) by *M. phaseolina* destroys the plant. The leaves wilt, lower stem often slough and exhibits shy-gray discoloration to form numerous small charcoal-black fruiting bodies (microsclerotia) and the plant dies (Bhattacharya *et al.,* 1994; Vasebi *et al.,* 2013).

As response to pathogen infestation, plant defensive mechanism triggers and causes the accumulation of reactive oxygen species (ROS). Higher production of ROS in response to disease is common stress that may be damaging in case of susceptible host and protective in resistant plant through the signaling factors (Gill and Tuteja, 2010; De Gara *et al.,* 2010). This alteration results great variability in the health markers (total chlorophyll content and carotenoids) and the stress markers including induction of protein synthesis, antioxidant enzymes (peroxidase, polyphenol oxidase and catalase) and total phenolics accumulation (Sharma *et al.,* 2012). Therefore, such markers would ideally be mechanistically associated with resistance acquired by host against invading pathogen.

Being a very important economical crop it is necessary to screen out resistant genotypes of soybean before having introducing them in the market. Therefore, present study was aimed to screen out resistant soybean genotype against *M. phaseolina* in response to charcoal rot disease on the basis of the disease, physiology and growth attributes.

MATERIALS AND METHODS

Preparation of *M. phaseolina* inoculum

M. phaseolina (FCBP # 0751) was sub-cultured in synthetic medium (2 % malt extract agar) in Petri dishes. After incubation at 28 ± 2 °C for 7 days, fungal sclerotial suspension was prepared for the pathogenicity trail, sclerotia per mL were counted through haemocytometer for the pathogenicity trail.

Procurement of soybean genotypes

The most cultivated five soybean genotypes viz., AJMERI, PSC-60, RAWAL-I, NARC-I and NARC-II were procured from National Agricultural Research Council (NARC), Islamabad, Pakistan. Sodium hypochlorite (2%) was used for seeds surface sterilization for one minute, followed by consecutive washings with distilled water and dried out for further use.

Experiment

Sandy loam soil was sterilized with 2% formalin solution (Khan *et al.,* 2016). Sterilized soil (4 kg pot^{-1}) was filled in the plastic pots (17.5 and 15 cm in length and width) and was inoculated with 15 mL pot^{-1} freshly prepared cultural suspension of *M. phaseolina*. Seven surface-sterilized seeds of each five different genotypes were sown in the pots kept in the tunnel during June-July in Kharif season, 2016 under completely randomized design (CRD). Pots without fungal inoculation (control) were also prepared with three replications, consisting of each five genotypes. The data regarding assessment of disease, physiology and growth of plants was recorded after 45days of sowing.

Disease assessment

For disease assessment, plants were regularly monitored for the appearance of visible symptoms. Abawi and Pastor-Corrales (1990) disease rating scale was used for determination the diseases severity. Disease incidence (DI) and plant mortality were recorded. The rate of disease incidence was calculated according to the formula described by Cohen *et al.* (2000).

$$\text{DI (\%)} = \frac{\text{Number of infected plants}}{\text{Total nutmber of plants}} \times 100$$

Physiological and biochemical parameters assessment

Total chlorophyll content and carotenoids

Fresh weight of soybean leaf samples (each 0.5g) were taken for each treatment. The plant extraction was prepared in 80% ethanol to assess total chlorophyll content and carotenoids. The absorbance for chlorophyll a (645 nm), chlorophyll b (663 nm) and carotenoids (270 nm) was determined (Lichtenthaler and Buschmann, 2001).

Total phenolics

Total phenolic content were quantified according to the modified method of Bray and Thorpe (1954). The plant tissue (1 g) was homogenized in 80% ethanol, centrifuged at 10,000 rpm for 10 minutes. The reaction mixture was containing 0.5 mL of ethanolic extract; 2.5 mL of 20% Na_2CO_3 along with 0.025 mL of Folin-Ciocalteatou's reagent was added. After incubation of samples at 45^{o}C for 45 min in thermostat, the absorbance was determined using spectrophotometer at 765 nm. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed.

Total protein content and antioxidant enzymes

The quantitative analysis of total protein contents was carried out by using method of Lowry *et al.* (1951). The peroxidase activity was assayed by the method of Kumar and Khan (1982). The polyphenol oxidase activity was analyzed according to Mayer *et al.* (1965). The activity of catalase was determined by modified method of Aebi (1974).

Growth attributes

Plant height and weight (fresh and dry weight) were used to calculate the growth inhibition index (GII) (Khan *et al.,* 2016).

Statistical analysis

Twenty-one plants in triplicate set (7 pot^{-1}) were analyzed individually. The data regarding plant's disease incidence, mortality, physiological, biochemical and growth parameters were subjected to analyze the variance

(ANOVA). Least significant difference test (LSD) at level of $P \leq 0.05$ was applied to compare the differences among means using software Statistics 8.1. Two sample T-Test was applied on the growth parameters to check difference in growth reduction in inoculated treatment with respect to un-inoculated treatment.

RESULTS

Diseases incidence and growth

In resistant group (AJMERI), no symptoms were observed and the plants were placed in category "1" (disease rating scale). The plants in this group showed the highest germination rate (81%) with the lowest disease incidence (16%), mortality (5%) and growth inhibition index (4%) (Table 1).

PSC-60 and RAWAL-I were found in moderately resistant group (MR) with the disease rating score "5". Both genotypes exhibited similar variation in the studies attributes against *M. phaseolina* except germination percentage. PSC-60 and RAWAL-I exhibited 62% and 76% germination, respectively. Disease incidence and mortality were about 40% and 20%, respectively with and growth inhibition index (GII) was 15% in both genotypes (Table 1).

Genotype NARC-II was categorized as susceptible (SS) and kept in the category "7". This genotype showed statistically similar germination percentage to the resistant group (81%), but with significantly greater mortality rate (34%) and disease incidence (61%) (Table 1). Cumulative GII was 26% (Table 1) and inhibition in growth parameters was ranged between 22-33% over their respective control (Table 3).

Soybean genotype NARC-I was found to be highly susceptible (disease rating score "9") to the effects of *M. phaseolina*. The germination rate was significantly lower (57%) due to the high disease severity and plants exhibited the maximum reduction in growth parameters (34-50%) that were significantly different from their respective control (Table 2; Table 3). Disease incidence, mortality and GII were significantly higher was maximum 76%, 50% and 43%, respectively as compared to other groups (Table 1).

Table 1. The response of different soybean genotypes to *Macrophomina phaseolina* on the germination and disease.

Genotypes	Groups	Germination	Disease incidence	Disease severity	Plant mortality	Growth inhibition index
				(%)		
AJMERI	Resistant (RR)	81 A	9 D	1	18 C	4 D
PSC-60	Moderately resistant	62 B	38 C	5	23 BC	15 C
RAWAL-I	(MR)	76 A	43 C	5	24 BC	15 C
NARC-II	Susceptible (SS)	81 A	61 B	7	34 B	26 B
NARC-1	Highly susceptible (HS)	57 B	76 A	9	50 A	43 A

Letters in a column show the significant differences ($P \leq 0.05$) in triplicate mean values as determined by LSD test.

Table 2. The effect of *Macrophomina phaseolina* on growth parameters of different soybean genotypes.

Genotypes	Shoot length (cm)		Shoot fresh weight (g)		Shoot dry weight (g)		Root length (cm)		Root fresh weight (g)		Root dry weight (g)	
	UI	I	UI	I	UI	I	UI	I	UI	I	UI	I
AJMERI	47.02	45.67	2.75	2.72	0.66	0.65	23.26	22.56	0.24	0.24	0.07	0.06
PSC-60	34.10	28.68*	1.77	1.43	0.44	0.34	16.71	14.18	0.21	0.19	0.05	0.05
RAWAL-I	46.13	43.33	1.73	1.46	0.42	0.36	22.81	21.47	0.12	0.08	0.03	0.02
NARC-II	37.33	29.33**	1.09	0.85**	0.28	0.21*	18.47	14.44*	0.09	0.06**	0.02	0.01**
NARC-1	28.67	19.00**	1.8	0.92**	0.43	0.23**	14.13	9.30**	0.11	0.06*	0.03	0.02*

* significant at $P \leq 0.05$ and ** significant at $P \leq 0.001$ according to two sample t-test comparing healthy (Uninoculated: UI) and inoculated (I) plants.

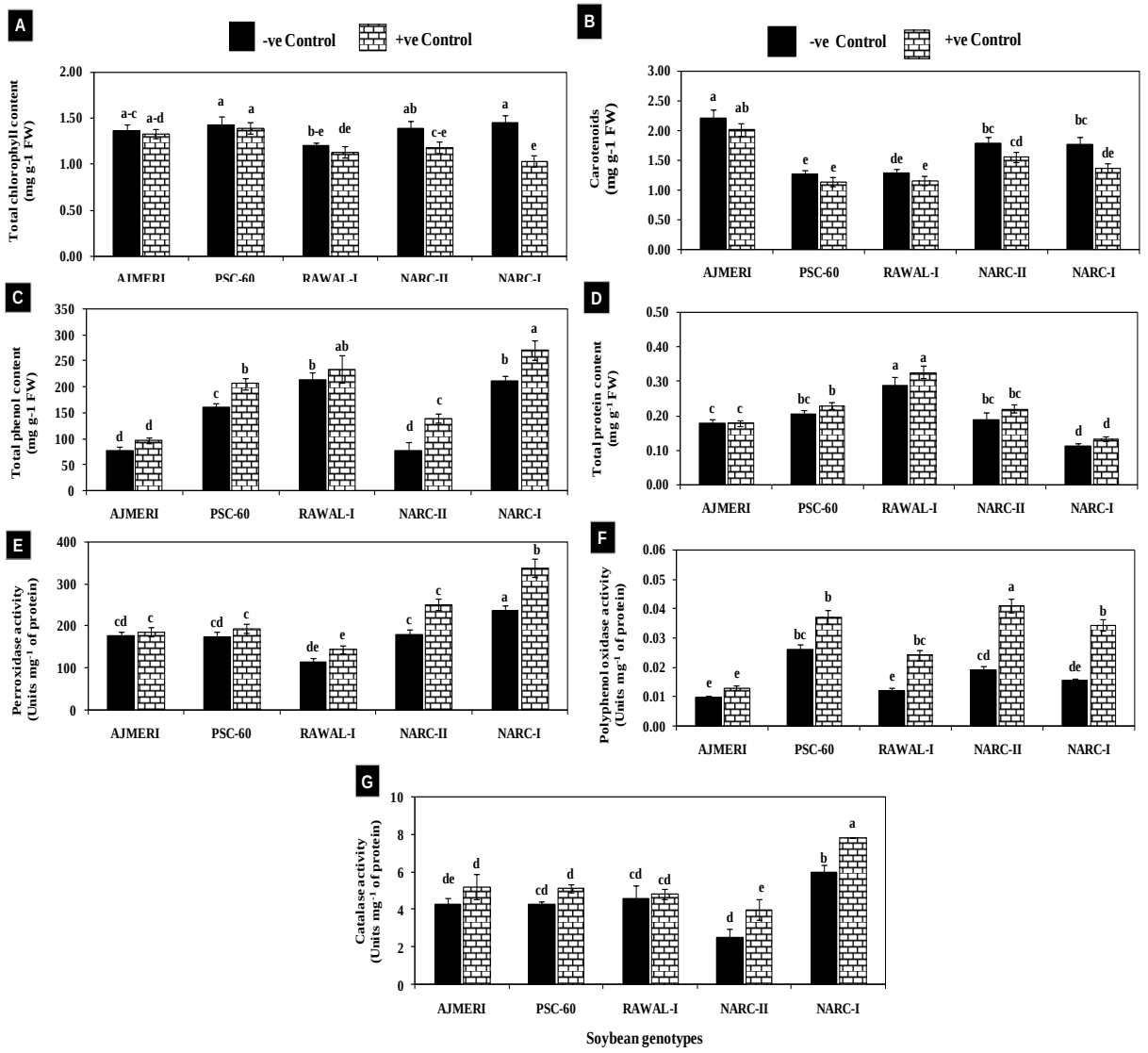

Fig. 1 (A-G). The effect of *Macrophomina phaseolina* (MP) on physiological parameters and antioxidant enzymes of soybean. Values with different letters at their top show significant difference (P = 0.05) as determined by LSD Test. Vertical bars show standard errors of means of three replicates.
-ve control (Uninoculated) and + ve control (inoculated with MP).

Table 3. The effect of *Macrophomina phaseolina* on % inhibition in different growth parameters of soybean genotypes.

Genotypes	Shoot length	Shoot fresh weight	Shoot dry weight	Root length	Root fresh weight	Root dry weight
	Inhibition (%)					
AJMERI	3±6.2 C	2±6.9 C	2±3.0 C	3±8.6B	1±3.7C	12±3.5 B
PSC-60	16±2.6 BC	21±3.9 B	22±8.5 B	15±6.2 AB	11±9.7 BC	11±9.2 B
RAWAL-I	6±5.1 C	15±1.5 BC	15±6.0 BC	6±8.7 B	32±7.2 AB	30±9.5 AB
NARC-II	22±3.2 AB	23±3.5 B	24±5.2 B	22±3.2 AB	33±6.4 AB	33±5.9 AB
NARC-1	34±6.1 A	49±4.0 A	48±7.2A	34±3.5 A	44±7.8A	49±10.2 A

Letters in each column show significant differences (P ≤ 0.05) in triplicate mean values as determined by LSD test.
± show standard errors of means of three replicates.

Plant physiology and biochemistry

Total chlorophyll content, carotenoids and phenolic content were insignificantly different in all three resistant genotypes (AJMERI, PSC-60 and RAWAL-I) as compared to their corresponding control treatments. Both in susceptible and highly susceptible groups total chlorophyll content and carotenoids were significantly decreased to 14-28% over their respective control. However, total phenolic content were significantly increased in susceptible and highly susceptible groups by 30% and 80%, respectively (Fig. 1 A-C). There was an insignificant difference in total protein content of all genotypes as compared to their respective control treatment (Fig. 1 D).

The current study also revealed the notably higher activities of defense related proteins against *M. phaseolina* charcoal rot pathogen. POX, PPO and CAT activities were estimated in leaves from pathogen inoculated (positive control) and un-inoculated (negative control) soybean plants. As the health markers, the activities of POX PPO and CAT were insignificantly affected in all three resistant genotypes. By contrast, the activities of these defensive enzymes were found to increase rapidly after inoculation. POX activities were significantly increased by 39% and 43% in susceptible and highly susceptible groups, PPO was significantly raised up to two folds in susceptible and highly susceptible groups by 114% and 124% and CAT was significantly high in susceptible groups by 30% and 60% about their relevant control (Fig. 1 E, F and G).

DISCUSSION

Screening of soybean for resistant genotypes is an appropriate and short term method to avoid the significant losses in the breeding program. In current study, five different genotypes of soybean were selected to assess their resistance level against *M. phaseolina*. Genotypes were categorized into four groups as resistant (AJMERI), moderately resistant (PSC-60 and RAWAL-I), susceptible (NARC-II) and highly susceptible (NARC-I) on the basis of disease rating scale (Abawi and Pastor-Corrales, 1990).

Response of soybean genotypes against the pathogen infection was different with respect to disease severity. No remarkable disease symptoms were observed in resistant (AJMERI) and moderately resistant (PSC-60 and RAWAL-I) genotypes. However, susceptible (NARC-II) and highly susceptible (NARC-I) genotypes showed visible disease symptoms after 20-24 days of seed sowing. Leaves were yellow in color and smaller than normal size. Collar region of the plants showed light gray discoloration, while stems and roots became brown to black in color. Disease caused loss of plant vigor, wilting leading to premature plant death. Numerous, small microsclerotia were seen on the epidermal tissue of the lower stems and roots of dead plants (Gupta and Chauhan, 2005).

Inoculated genotypes kept in resistant and moderately resistant groups did not show significant alterations in growth, physiological and biochemical attributes. However, the investigated parameters were significantly and drastically affected in susceptible and highly susceptible groups. Chlorophyll ‘a’, chlorophyll ‘b’ and carotenoids were significantly decreased in both susceptible and highly susceptible genotypes as compared to their un-inoculated healthy plants. However, total phenolic content and antioxidant enzymes (POX, PPO and CAT) were significantly increased in these two genotypes. In susceptible and highly susceptible groups, pathogen infestation might be responsible for causing oxidative damage due to oxidation of DNA, proteins and lipids, leading to cell death (Gill and Tuteja, 2010). Damage to DNA in response to over production of ROS affects in all plants (Gill and Tuteja, 2010; Sharma *et al.,* 2012). Intensification in total phenolic content and the enzyme activities in susceptible and highly susceptible groups although indicated the activation of the host defense mechanism against stress by scavenging ROS. However, it seems that the antioxidants were not able to control the amount of ROS to an extent that could be helpful in relieving stressful conditions in susceptible and highly susceptible groups (Akinson and Urwi, 2012). Therefore overall changes in the health and stress markers in susceptible groups led to significant reduction in the growth parameters by 20-50% with a reduction in the cumulative growth inhibition index.

The current study concluded that high infestation of *M. phaseolina* infection altered the growth, plant physiology (total chlorophyll content and carotenoids) and biochemical attributes (total phenolic content, total protein content, POX, PPO and CAT) in susceptible and highly genotypes. Based on pathogenicity test, screening of soybean genotypes for identification of resistance against charcoal rot disease in potted soil is found to be appropriate and short term method.

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