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Changes in Phenolics and Some Oxidative Enzymes in Fenugreek Leaves Due to Powdery Mildew Infection

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The contents of total phenols and orthodihydric phenols, specific activities of polyphenol oxidase (PPO), peroxidase (PO) and catalase in leaves of powdery mildew resistant (NLM and HM 350) and susceptible (T 8 and HM 65) genotypes of fenugreek were estimated at 40, 80 and 100 days after sowing (DAS) in inoculated (E_1) and natural (E_2) environments. The levels of all the biochemical constituents were higher in resistant genotypes than in susceptible ones before the appearance of disease (40 DAS) in both the environments. In response to infection, an increase was observed in contents of all the parameters except catalase activity in all the genotypes. However, at higher disease severity levels of all the biochemical parameters decreased invariably in all the genotypes except activities of PPO and PO which increased further in susceptible genotypes in both the environments. The role of phenolics and oxidative enzymes in determining resistance in fenugreek against powdery mildew disease has been highlighted.

Keywords: Fenugreek, enzyme activity, phenolics, powdery mildew, resistance.

Fenugreek (*Trigonella foenum-graecum* L.) is an important legume crop grown in India during winter for multifarious uses like seed, fodder, vegetable, spices, etc. The importance of its seeds has further increased due to presence of an important steroid 'diosgenin' which is used in synthesis of sex hormones and oral contraceptives (Banyai, 1973). Inspite of multiple uses, the productivity of fenugreek is, however, low in India because of the losses caused due to biotic stresses such as fungi, bacteria and viruses. Fungal diseases particularly powdery mildew caused by *Erysiphe polygoni* DC alone accounts for more than 50 per cent losses in seed yield (Rathi et al., 2000).

The phenolic compounds, which are antibiotic in nature, have been found in all the plants investigated to date (Nicholson and Hammerschmidt, 1992). These compounds are toxic to certain fungi and bacteria (Kosuge, 1969) and their appearance is considered as part of an active defence response. These compounds are involved in resistance mechanism (Farkas and Király, 1962) and their concentration is much higher in resistant genotypes than in susceptible ones at all the growth stages (Parashar and Sindhan, 1986; Kalia and Sharma, 1988; Chander, 1989; Rathi et al., 1998).

Oxidative enzymes such as peroxidase (PO) and polyphenol oxidase (PPO) confer resistance to pathogens by oxidation of phenolic compounds (Sempio et al., 1975), which are more toxic than phenols themselves. These enzymes are also involved in yielding

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lignin like substances which can act as a barrier to further spread of the invading pathogen and generally their activity is higher in resistant cultivars (Kalia and Sharma, 1988; Sherif et al., 1989; Chander, 1990). In fenugreek, the information on these biochemical parameters governing resistance against powdery mildew is non-existent. Therefore, the present investigation was carried out to gather information on quantitative changes in phenolic compounds and specific activities of PPO, PO and catalase in healthy and diseased leaves of resistant and susceptible genotypes of fenugreek to powdery mildew infection at different growth stages.

Materials and Methods

Four genotypes of fenugreek, of which two were resistant (NLM and HM 350) and two were susceptible (T 8 and HM 65) to powdery mildew, were grown in RBD with three replications under two different environments i.e. artificially inoculated with conidia (E_1) and natural or uninoculated (E_2) at research farm of CCS Haryana Agricultural University, Hisar. The soil of the experiment plots was sandy loam in texture, low in organic carbon (0.28%) and available nitrogen (170 kg N ha^{-1}), medium in available phosphorus (20 kg P_2O_5 ha^{-1}) and rich in potash content (320 kg K_2O ha^{-1}) having Ece 0.30 dS m^{-1} and slightly alkaline in reaction (pH 7.7). Recommended dose of fertilizers (20 kg N and 40 kg P_2O_5 ha^{-1}) was applied before sowing.

For biochemical analysis, leaf samples were collected from all the genotypes in both E_1 and E_2 at three different growth stages i.e. 40 days after sowing (before appearance of disease), 80 DAS (at disease initiation) and 100 DAS (when disease was in severe form). From resistant genotypes, leaf samples collected were healthy (having no visible symptoms of the disease) at all the stages, whereas samples collected from susceptible genotypes were categorized into three groups i.e. healthy, 50% diseased and 100% diseased.

The leaf samples were thoroughly washed, finely chopped and 1.0 g of each category was ground with sea sand in a previously chilled mortar with 4–5 ml of 0.01 M phosphate buffer (pH 7.0). The homogenate was strained through a four layered cheese cloth. The filtrate was centrifuged at 10,000 g for 20 min. at 40 °C and the supernatant was used for enzyme assays. In each case three separate extractions were made. PPO and PO were assayed according to the modified methods of Taneja and Sachar (1974) and Shannon et al. (1966), respectively. Enzyme units were expressed as change in 0.01 absorbance min^{-1} mg^{-1} protein. Catalase activity was measured by following the procedure of Sinha (1972) and expressed as μ moles of H_2O_2 utilized min^{-1} mg^{-1} protein. Suitable blanks in each case were also run simultaneously. Soluble protein in the enzyme extract was precipitated by TCA and determined as per Lowry et al., (1951).

Leaf samples after the extraction of enzymes, were dried at 60 °C in a hot air oven and ground in a micro-Willey mill. Total phenols were then extracted and estimated by the method of Swain and Hillis (1959), whereas ortho-dihydric phenols were estimated by following the procedure given by Johnson and Schaal (1952).

Results

Total phenols and ortho-dihydric phenols

The resistant genotypes exhibited higher contents of total and ortho-dihydric phenols in comparison to healthy and diseased leaves of susceptible genotypes at all the growth stages in both the environments *(Tables 1 and 2).* The contents of both the phenolic compounds increased from 40 to 80 days after sowing (DAS) and then a slight decrease was observed from 80 to 100 DAS in both E_1 and E_2. In case of susceptible genotypes, healthy leaves contained minimum contents of these phenolics, whereas 100% diseased leaves exhibited maximum contents in both the environments. Moreover, in E_1 at 100 DAS, all the genotypes contained considerably higher contents of both these compounds in comparison to E_2, whereas no such trend for E_1 and E_2 was observed at 40 and 80 DAS.

Table 1

Total phenols (mg g^{-1}) in healthy (H) and diseased (D) fenugreek leaves of resistant and susceptible genotypes in two environments at different growth stages

Genotype	40 DAS		80 DAS		100 DAS	
	E_1	E_2	E_1	E_2	E_1	E_2
Resistant						
NLM (H)	36.61 ± 0.38	36.30 ± 1.27	44.80 ± 0.48	45.03 ± 0.30	42.14 ± 0.23	38.93 ± 0.41
HM 350 (H)	36.77 ± 0.17	37.65 ± 0.87	44.23 ± 0.19	44.49 ± 0.32	41.62 ± 0.99	39.24 ± 0.26
Susceptible						
T 8 (H)	29.73 ± 0.50	29.27 ± 0.48	31.85 ± 0.31	31.74 ± 0.82	31.67 ± 0.73	29.62 ± 0.47
T 8 (50% D)	–	–	35.78 ± 0.64	36.75 ± 1.36	32.31 ± 0.13	31.08 ± 0.11
T 8 (100% D)	–	–	39.18 ± 0.68	39.03 ± 0.28	36.85 ± 0.18	33.40 ± 0.54
HM 65 (H)	30.98 ± 0.63	31.33 ± 0.69	32.11 ± 0.33	32.25 ± 0.29	31.18 ± 0.51	30.80 ± 0.19
HM 65 (50%D)	–	–	35.44 ± 0.41	35.13 ± 0.63	34.98 ± 0.18	32.01 ± 0.08
HM 65 (100% D)	–	–	37.78 ± 0.18	38.17 ± 0.53	36.76 ± 0.46	34.50 ± 0.09
CD at 5%	–	–	2.13	1.98	1.77	2.09

DAS = Days after sowing
E_1 = Inoculated environment
E_2 = Natural environment

Polyphenol oxidase, peroxidase and catalase activity

The activities of PPO and PO were significantly higher in resistant genotypes than in healthy leaves of susceptible genotypes at 40 DAS in both E_1 and E_2 *(Tables 3 and 4).* There was an increase in the activities of both PPO and PO in all the genotypes at 80 DAS in both the environments and the increase was more pronounced in more infected leaves of susceptible genotypes. At 100 DAS, the activities decreased in resistant genotypes, whereas in susceptible ones it further increased in both E_1 and E_2. The activities of both

PPO and PO were slightly higher in resistant and lower in susceptible genotypes in E_2 as compared to E_1 at 100 DAS, while at 40 and 80 DAS differences between E_1 and E_2 were not remarkable.

Table 2

Ortho-dihydric phenols (mg g^{-1}) in healthy (H) and diseased (D) fenugreek leaves of resistant and susceptible genotypes in two environments at different growth stages

Genotype	40 DAS		80 DAS		100 DAS	
	E_1	E_2	E_1	E_2	E_1	E_2
Resistant						
NLM (H)	6.37 ± 0.04	6.54 ± 0.06	7.61 ± 0.04	7.54 ± 0.02	6.46 ± 0.09	6.22 ± 0.06
HM 350 (H)	6.42 ± 0.03	6.37 ± 0.04	8.53 ± 0.12	8.37 ± 0.03	7.12 ± 0.05	6.34 ± 0.07
Susceptible						
T 8 (H)	4.28 ± 0.04	4.41 ± 0.07	4.38 ± 0.02	4.44 ± 0.06	4.30 ± 0.04	3.82 ± 0.05
T 8 (50% D)	–	–	5.69 ± 0.06	5.88 ± 0.07	5.14 ± 0.06	5.08 ± 0.10
T 8 (100% D)	–	–	6.30 ± 0.06	6.26 ± 0.13	5.90 ± 0.06	5.49 ± 0.08
HM 65 (H)	4.91 ± 0.07	4.87 ± 0.05	4.89 ± 0.02	4.97 ± 0.06	4.13 ± 0.07	3.63 ± 0.03
HM 65 (50%D)	–	–	5.61 ± 0.10	5.53 ± 0.02	5.25 ± 0.16	4.77 ± 0.12
HM 65 (100% D)	–	–	5.88 ± 0.04	5.94 ± 0.04	5.75 ± 0.14	4.93 ± 0.09
CD at 5%	–	–	0.27	0.34	0.37	0.23

Mean values of triplicate determinations.
DAS = Days after sowing
E_1 = Inoculated environment
E_2 = Natural environment

Table 3

Specific activity* of polyphenol oxidase in healthy (H) and diseased (D) fenugreek leaves of resistant and susceptible genotypes in two environments at different growth stages

Genotype	40 DAS		80 DAS		100 DAS	
	E_1	E_2	E_1	E_2	E_1	E_2
Resistant						
NLM (H)	1.34 ± 0.04	1.31 ± 0.08	1.40 ± 0.01	1.42 ± 0.03	0.97 ± 0.01	1.09 ± 0.01
HM 350 (H)	1.38 ± 0.05	1.41 ± 0.10	1.43 ± 0.04	1.49 ± 002	1.05 ± 0.01	1.12 ± 0.03
Susceptible						
T 8 (H)	0.84 ± 0.01	0.86 ± 0.01	1.34 ± 0.06	1.32 ± 0.09	1.89 ± 0.08	1.72 ± 0.03
T 8 (50% D)	–	–	1.38 ± 0.02	1.43 ± 0.02	2.01 ± 0.13	1.92 ± 0.08
T 8 (100% D)	–	–	1.61 ± 0.08	1.54 ± 0.06	2.16 ± 0.11	2.05 ± 0.10
HM 65 (H)	0.91 ± 0.04	0.95 ± 0.01	1.28 ± 0.01	1.33 ± 0.06	1.94 ± 0.07	1.75 ± 0.09
HM 65 (50%D)	–	–	1.44 ± 0.04	1.45 ± 0.03	2.10 ± 0.03	1.86 ± 0.04
HM 65 (100% D)	–	–	1.60 ± 0.01	1.58 ± 0.04	2.19 ± 0.06	2.12 ± 0.05
CD at 5%	–	–	0.17	0.14	0.21	0.19

*Enzyme units – Change in 0.01 O.D. min^{-1} mg^{-1} protein
DAS = Days after sowing
E_1 = Inoculated environment
E_2 = Natural environment

Table 4

Specific activity* of peroxidase in healthy (H) and diseased (D) fenugreek leaves of resistant and susceptible genotypes in two environments at different growth stages

Genotype	40 DAS		80 DAS		100 DAS	
	E_1	E_2	E_1	E_2	E_1	E_2
Resistant						
NLM (H)	$2.28 \times 10^3 \pm 0.13 \times 10^3$	$2.30 \times 10^3 \pm 0.09 \times 10^3$	$3.42 \times 10^3 \pm 0.07 \times 10^3$	$3.35 \times 10^3 \pm 0.03 \times 10^3$	$1.21 \times 10^3 \pm 0.07 \times 10^3$	$1.38 \times 10^3 \pm 0.08 \times 10^3$
HM 350 (H)	$2.56 \times 10^3 \pm 0.13 \times 10^3$	$2.69 \times 10^3 \pm 0.14 \times 10^3$	$3.89 \times 10^3 \pm 0.02 \times 10^3$	$4.03 \times 10^3 \pm 0.09 \times 10^3$	$1.08 \times 10^3 \pm 0.09 \times 10^3$	$1.56 \times 10^3 \pm 0.10 \times 10^3$
Susceptible						
T 8 (H)	$1.58 \times 10^3 \pm 0.04 \times 10^3$	$1.52 \times 10^3 \pm 0.07 \times 10^3$	$2.96 \times 10^3 \pm 0.12 \times 10^3$	$2.88 \times 10^3 \pm 0.05 \times 10^3$	$4.87 \times 10^3 \pm 0.18 \times 10^3$	$4.20 \times 10^3 \pm 0.14 \times 10^3$
T 8 (50% D)	–	–	$3.75 \times 10^3 \pm 0.08 \times 10^3$	$3.66 \times 10^3 \pm 0.11 \times 10^3$	$6.66 \times 10^3 \pm 0.13 \times 10^3$	$6.09 \times 10^3 \pm 0.31 \times 10^3$
T 8 (100% D)	–	–	$4.27 \times 10^3 \pm 0.07 \times 10^3$	$4.36 \times 10^3 \pm 0.08 \times 10^3$	$9.36 \times 10^3 \pm 0.21 \times 10^3$	$8.81 \times 10^3 \pm 0.06 \times 10^3$
HM 65 (H)	$1.53 \times 10^3 \pm 0.03 \times 10^3$	$1.41 \times 10^3 \pm 0.05 \times 10^3$	$2.43 \times 10^3 \pm 0.01 \times 10^3$	$2.43 \times 10^3 \pm 0.05 \times 10^3$	$4.23 \times 10^3 \pm 0.15 \times 10^3$	$3.75 \times 10^3 \pm 0.29 \times 10^3$
HM 65 (50%D)	–	–	$3.24 \times 10^3 \pm 0.06 \times 10^3$	$3.32 \times 10^3 \pm 0.01 \times 10^3$	$6.32 \times 10^3 \pm 0.09 \times 10^3$	$5.87 \times 10^3 \pm 0.14 \times 10^3$
HM 65 (100% D)	–	–	$4.10 \times 10^3 \pm 0.18 \times 10^3$	$4.04 \times 10^3 \pm 0.04 \times 10^3$	$8.54 \times 10^3 \pm 0.12 \times 10^3$	$7.98 \times 10^3 \pm 0.18 \times 10^3$
CD at 5%	–	–	0.33×10^3	0.38×10^3	0.73×10^3	0.58×10^3

*Enzyme units – Change in 0.01 O.D. min^{-1} mg^{-1} protein
DAS = Days after sowing
E_1 = Inoculated environment
E_2 = Natural environment

The activity of catalase was higher in resistant genotypes as compared to all categories of susceptible genotypes at all the growth stages in both E_1 and E_2 *(Table 5).* The activity of this enzyme was at peak at 40 DAS and decreased thereafter at 80 and 100 DAS in all the genotypes in both the environments. Moreover, catalase activity was also higher at 100 DAS in E_2 in all the genotypes than in E_1, whereas at 40 and 80 DAS, differences in E_1 and E_2 were generally not considerable.

Discussion

Phenolic compounds act as preformed resistance factors and are generally considered as most responsible parameters for diseases resistance (Sathiyanathan and Vidhyasekaran, 1981). Also phenolics accumulation is generally higher in resistant genotypes than in susceptible ones (Parashar and Sindhan, 1986; Kalia and Sharma 1988; Chander, 1989; Rathi et al., 1998). The results of the present study are also in accordance with these findings. In the defence response to pathogen, the contents of both the phenolics increased in all the geno-

Table 5

Specific activity* of catalase in healthy (H) and diseased (D) fenugreek leaves of resistant and susceptible genotypes in two environments at different growth stages

Genotype	40 DAS		80 DAS		100 DAS	
	E_1	E_2	E_1	E_2	E_1	E_2
Resistant						
NLM (H)	$2.48 \times 10^3 \pm 0.13 \times 10^3$	$2.39 \times 10^3 \pm 0.11 \times 10^3$	$2.13 \times 10^3 \pm 0.06 \times 10^3$	$2.18 \times 10^3 \pm 0.02 \times 10^3$	$1.71 \times 10^3 \pm 0.04 \times 10^3$	$1.92 \times 10^3 \pm 0.08 \times 10^3$
HM 350 (H)	$2.50 \times 10^3 \pm 0.09 \times 10^3$	$2.51 \times 10^3 \pm 0.01 \times 10^3$	$2.15 \times 10^3 \pm 0.07 \times 10^3$	$2.10 \times 10^3 \pm 0.03 \times 10^3$	$1.63 \times 10^3 \pm 0.13 \times 10^3$	$1.96 \times 10^3 \pm 0.04 \times 10^3$
Susceptible						
T 8 (H)	$1.21 \times 10^3 \pm 0.07 \times 10^3$	$1.27 \times 10^3 \pm 0.04 \times 10^3$	$1.24 \times 10^3 \pm 0.12 \times 10^3$	$1.12 \times 10^3 \pm 0.06 \times 10^3$	$0.72 \times 10^3 \pm 0.06 \times 10^3$	$0.84 \times 10^3 \pm 0.04 \times 10^3$
T 8 (50% D)	–	–	$0.89 \times 10^3 \pm 0.01 \times 10^3$	$0.92 \times 10^3 \pm 0.02 \times 10^3$	$0.41 \times 10^3 \pm 0.02 \times 10^3$	$0.61 \times 10^3 \pm 0.03 \times 10^3$
T 8 (100% D)	–	–	$0.76 \times 10^3 \pm 0.05 \times 10^3$	$0.80 \times 10^3 \pm 0.03 \times 10^3$	$0.30 \times 10^3 \pm 0.01 \times 10^3$	$0.45 \times 10^3 \pm 0.02 \times 10^3$
HM 65 (H)	$1.41 \times 10^3 \pm 0.10 \times 10^3$	$1.32 \times 10^3 \pm 0.05 \times 10^3$	$1.17 \times 10^3 \pm 0.02 \times 10^3$	$1.18 \times 10^3 \pm 0.09 \times 10^3$	$0.76 \times 10^3 \pm 0.05 \times 10^3$	$0.92 \times 10^3 \pm 0.09 \times 10^3$
HM 65 (50%D)	–	–	$0.96 \times 10^3 \pm 0.05 \times 10^3$	$0.98 \times 10^3 \pm 0.08 \times 10^3$	$0.52 \times 10^3 \pm 0.01 \times 10^3$	$0.59 \times 10^3 \pm 0.02 \times 10^3$
HM 65 (100% D)	–	–	$0.74 \times 10^3 \pm 0.02 \times 10^3$	$0.76 \times 10^3 \pm 0.03 \times 10^3$	$0.26 \times 10^3 \pm 0.01 \times 10^3$	$0.42 \times 10^3 \pm 0.01 \times 10^3$
CD at 5%	–	–	0.19×10^3	0.23×10^3	0.21×103	0.13×10^3

*Enzyme units – μ moles of H_2O_2 utilized min^{-1} mg^{-1} protein
DAS = Days after sowing
E_1 = Inoculated environment
E_2 = Natural environment

types and then decreased uniformly at 100 DAS. Sempio et al. (1975) advocated that resistance is expressed by oxidation of phenols to quinones, which are more toxic to microorganisms. In susceptible genotypes the fungus got enough time for its growth, whereas in resistant cultivars higher accumulation of phenols at initial stages might have succeeded in restriction of the pathogen. At 100 DAS, all the genotypes in E_1 exhibited higher contents of phenolic compounds than in E_2. This was because of the fact that disease intensity was more in E_1. Accumulation of phenols at higher levels might be the tendency of the host to isolate the pathogen at original site of ingress (Legrand, 1983; Ride, 1983).

Investigations have noted that PPO and PO confer resistance to pathogens by oxidation of phenolic compounds and yielding lignin like substances (Vance et al., 1980), which act as barriers to invading pathogens (Hijwegen, 1963). In the present study the activities of PPO and PO were observed to be higher in resistant genotypes than in susceptible ones before the appearance of the disease (40 DAS). As soon as disease appeared (80 DAS), the activity of both the enzymes increased in all the resistant and susceptible genotypes but this increase was more pronounced in susceptible genotypes in both E_1 and E_2. As the disease

progressed and reached at peak (100 DAS), the activities of these enzymes in resistant genotypes decreased, while in susceptible ones it further increased. Similar results were also obtained by Sangwan et al. (1996) for both PPO and PO; Wood and Barbara (1971), Gupta et al. (1990) and Gupta et al. (1995) for PO in different host-parasite interactions. The post-infectional increased activity of PPO and PO in susceptible genotypes might be required for additional deposition of lignin around the lesions induced by the pathogens.

The role of catalase in diseases resistance is still not much clear. The presence of this enzyme in higher concentration in resistant genotypes in comparison to susceptible ones at all the growth stages indicates its possible role towards resistance. However, after infection, catalase activity decreased uniformly in all the genotypes and was minimum at 100 DAS in both the environments. Similar findings have also been observed by Sherif et al. (1989); Gupta et al. (1995) and Sangwan et al. (1996) in various host-parasite combinations. However, findings of Singh and Mall (1974) and Sathiyanathan and Vidhyasekaran (1981) are in variance to the present investigation. They have further suggested that the increased catalase activity upon infection probably reduced the efficacy of natural defenses of plant through the suppression of PO activity.

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