Int J Seed Spice, 14(1), June, 2024: 81-88 DOI: https://doi.org/10.56093/IJSS.v14i1.9

Biochemical characterization of fenugreek (*Trigonella foenum-graecum* L.) genotypes for downy mildew resistance

M.K. Mahatma,Y.K. Sharma*, M.R. Sharma, R.S. Meena, C.K. Jangir and R.D. Meena

Abstract

Downy mildew (*Peronospora trigonella* Gaumann) of fenugreek is the most serious disease all over the country.The leaves of fenugreek genotypes AM-232, AM-251, AM-86, AM-256, AM-286AM-660, AM-661, AM-663, AM-651 AM-665 and AFg-1 were taken after 5 days of visual symptoms from the field for biochemical characterization. Biochemical data were correlated with percent disease index (PDI) score. Maximum PPO activity was observed in AM-85 (10.5 unit $min^{-1}g^{-1}$ protein) and AM-256 (8.5 unit $min^{-1}g^{-1}$ protein). Genotypes AM-85 and AM-256 had PDI score of 26.3 and 16.3, respectively for downy mildew disease. Catalase activity was observed maximum in AM-660 (17.5μmol H_2O_2 min^{-1} g^{-1} protein) followed by AM-660 and AM-232 with a PDI score of 21.3, 23.8 and 8.8 respectively. Peroxidase activity was found maximum in AM-651 (1102.6 μmol guaiacol min^{-1} g^{-1} protein) with a maximum PDI score (32) while minimum activity was observed in AM-256 (517μmol guaiacol min^{-1} g^{-1} protein). Total phenol concentration in fenugreek genotypes was observed from 1.1 to 4.0 mg^{-1} g FW . Genotypes with higher phenol content scored less PDI than those with lower phenol content. PCA-biplot and correlation analysis revealed a significant positive correlation between PDI score and peroxidase activity while a significant negative correlation of PDI score was observed with catalase(-0.769**), PPO activity (-0.754**) and phenol content (0.570*). Biochemical and PDI score data were further used to obtain hierarchial clustering of genotypes which clustered fenugreek genotypes into three groups. The first group consisted of AM-232 and AM-256 with a PDI score of 8.8 and 16.3, respectively and identified as resistant sources for downy mildew disease of fenugreek. Thus, the present study demonstrated that PPO, peroxidase and catalase activity along with phenol content can distinguish the resistant germplasm.

Keywords: Catalase, downy mildew, fenugreek, PPO, peroxidase, phenol

Reviewed by
Dr. Harsur M.Jajda
ASPEE SHAKILAM Biotechnology Institute,
Navsari Agricultural University, Surat, Gujarat, India

Email: harsurbiochem@gmail.com

***Correspondence**
Y.K. Sharma
yksharma68@gmail.com

Received: 10 December 2024
Revision: 09 January 2025
Accepted: 09 January 2025

Citation
Mahatma, M.K., Sharma, Y.K., Sharma, M.R., Meena, R.S., Jangir, C.K. and Meena, R.D. 2024. Biochemical characterization of fenugreek (*Trigonella foenum-graecum* L.) genotypes for downy mildew resistance. *Int J Seed Spice,* 14 (1): 81-88

DOI
https://doi.org/10.56093/IJSS.v14i1.9

Affiliation
[1]ICAR-National Research Centre on Seed Spices, Tabiji, Ajmer-305206, Rajasthan, India

Introduction

Fenugreek (*Trigonella foenum-graecum* L.) is an important leguminous spice grown worldwide for culinary and medicinal purpose. Fenugreek is an annual flowering plant, with autogamous flowers. It is native to the area extending from Iran to northern India and is widely cultivated in China, India, Egypt, Ethiopia, Morocco, Ukraine, Greece, Turkey, etc. (Petropoulos 2002). Fenugreek is a multipurpose crop grown for vegetable or herb (leaf), fodder and seed purpose. Fenugreek is prone to several fungal and bacterial diseases, among them downy mildew causes maximum crop loss. The occurrence of downy mildew was first reported in Bombay by Uppal *et al.* (1935). This disease causes rapid and enormous damage which ultimately limits successful and economical cultivation of fenugreek in India (Goyal and Sharma, 2012). Downy mildew not only reduce yield but also affects leaf and fodder quality. Downy mildews are caused by obligate oomycete pathogens belonging to the family Peronosporaceae within the order Peronosporales. Conidiospores typically appear as a white, fluffy and downy growth on a leaf's bottom or abaxial surface. This symptom gives the name “Downy” to the disease. Crop rotation, the use of contact and systemic insecticides, biopesticides, cultural methods, and the introduction of resistant cultivars have all been used historically to combat downy mildew disease (Tör *et al.*, 2023). However, the identification or development of resistant genotypes for disease resistance is a sustainable way to disease control.

Gene-for-gene interactions frequently condition plants to be resistant to disease. Therefore, the host plant quickly activates defense responses when the pathogen possesses an avirulence (Avr) gene and the host plant possesses its corresponding resistance (R) gene. Hypersensitive response is another mechanism of plant resistance to pathogens (Keen *et al.* 1981). Phenolic compounds play an important role in plant defense. Phenolic compounds are synthesized by phenylpropanoid pathway in which phenylalanine ammonia lyase is an important enzyme (Kumar *et al.*, 2014). There are numerous roles for peroxidases, such as the hypersensitive response, lignification, cross-linking of phenolic and glycoprotein, suberization, and phytoalexin synthesis. By oxidizing phenolic chemicals to harmful antimicrobial quinones, the polyphenol oxidase (PPO) enzyme plays a vital role in restricting the pathogen growth (Mahatma *et al.*, 2011). PPO is also known to play a major role in lignin biosynthesis. It has been established that during plant-pathogen interaction defense enzymes like phenylalanine ammonia lyase, peroxidase, polyphenol oxidase β-1,3-glucanase, chitinase, lipoxygenase, catalase are significantly higher in resistant genotypes. A pathogen's ingress is largely prevented by a timely and appropriate perception of it by the host cells and subsequent activation of its defense mechanisms. A coordinated expression of defense enzymes, secondary metabolites, and pathogen-related proteins is required to stop pathogens spread. Based on theese information, a study was conducted to monitor some of the biochemical changes in fenugreek leaves during downy mildew disease and to correlate those changes with disease resistance.

Materials and Methods

The leaves of fenugreek genotypes AM-232, AM-251, AM-86, AM-256 and AM-286 (Downy mildew resistant), AM-660, AM-661, AM-663, AM-651 and AM-665 (Downy mildew susceptible), AFg-1 (Check line) were taken from the field experiment of Plant Pathology Section, ICAR- National Research Centre on Seed Spices, Tabiji, Ajmer, Rajasthan, India. The crop was sown in 3 m row length with row and plant spacing of 30×10 cm. All genotype were sown in five intervening rows and genotype AFg-1 was sown after every fifth rows. All recommended package of practices were followed to raise the crop. The percent disease index (PDI) was calculated for each genotype for disease reaction.

Extraction and assay of enzymes activity

Extract for catalase (CAT), guaiacol peroxidase (GPOX), and polyphenol oxidase (PPO) activities was prepared from 0.5 g fresh leaves of control as well as infected plants. Upper third leaves were taken for biochemical determination after 5 days of visual symptoms of downy mildew disease (65 days after sowing). Leaves were homogenized in a pre-chilled mortar and pestle with 5 mL of an ice cold 50 mM phosphate buffer having pH 7.0 (Kapadia *et al.*, 2012). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatant was used for the assay. This supernatant was used for the assay of the catalase (CAT), guaiacol peroxidase (GPOX) and polyphenol peroxidise (PPO) following the method described by Hake *et al*. (2018). CAT (EC 1.11.1.6) activity was determined in 3 mL homogenates containing 50 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 100 µL enzyme extract by measuring the decrease in the absorption at 240 nm as H_2O_2 (ε=39.4 mM^{-1} cm^{-1}). GPOX (EC 1.11.1.7) activity was assayed in a 3 mL reaction mixture containing 50 mM sodium phosphate buffer pH 7.0, 0.1 mM EDTA, 50 µL enzyme extract, 10 mM guaiacol and 10 mM H_2O_2. Increase in absorption at 470 nm due to the formation of tetraguaiacol (ε = 26.6 mM^{-1} cm^{-1}) was recorded for 1 min at an interval of 15 second. Polyphenol oxidase (EC 1. 14.18.1) activity was determined from a reaction mixture containing 2.9 mL of catechol (0.01 M) in 10 mM phosphate buffer pH 6.0) and the reaction was initiated by the addition of 0.1 mL of enzyme extract. The change in the colour due to the oxidized catechol was observed at 490 nm on spectrophotometer for one minute at an interval of 15 second. The Molar extinction coefficient for catechol is 25.5 mM^{-1} cm^{-1}. CAT, GPOX and PPO was expressed as mmol substrate oxidised min^{-1} g^{-1} protein. Protein content of enzyme extract was estimated by lowery method (Lowry *et al.*,1951).

Metabolite constituents

Total Sugars, free amino acids and phenol content

Total phenol, toal sugar and free amino acids were extracted with 80% ethanol. Total soluble sugars were estimated using 0.2% anthrone reagent (200 mg in 100 mL H_2SO_4). The intensity of colour was read at 600 nm on a spectrophotometer. A standard curve was prepared using 10 mg glucose per 100 mL distilled water (Franscistt *et al.*, 1971). Total phenol content was estimated with Folin & Ciocalteu reagent as described by Malik and Singh (1980) and standard graph was prepared using gallic acid. Free Amino Acids were determined by using ninhydrin (Lee and Takahanshi, 1966). Ethanol-extracted supernatant (0.4 mL) was taken in a test tube and volume made up 1 mL by adding distilled water. 5 mL of ninhydrin reagent was prepared by mixing 1% ninhydrin (dissolved in 0.5 M citrate buffer pH 5.5): Glycerol: 0.5 M Citrate buffer pH 5.5 in the ratio of 5:12:2. The tubes were placed in a water bath at 100 C for 12 minutes to develop colour and cooled at room temperature under running water. The intensity of colour was read at 570 nm against the blank prepared by adding 0.4 mL 80% ethanol in place of extract. A standard curve was prepared by glycine in the range of 0-80 µg concentration.

Statistical analysis

All the biochemical parameters were analyzed in triplicates. The data obtained by biochemical constituents and enzyme determination were subjected to a simple completely randomized design (CRD) for the study of the significance of various data. PCA-biplot and correlation analysis were done using R-software.

Results and discussion

Disease incidence score and symptoms

Downy mildew disease incidence clearly distinguished fenugreek genotypes in resistant and susceptible categories (Table 1). Minimum disease incidence was observed in genotype AM-232 (8.8%) while maximum in AM-651 (40%) followed by in AM-665 (32.5%). Downy mildew infection in fenugreek manifests with a combination of symptoms on lower and upper leaf surface (Fig 1). Fungal mycelium of the downy mildew pathogen was observes on the underside of the infected leaves as a grayish-white, fuzzy growth. While on the upper leaves Irregular yellow spots appeared.

Table 1. Metabolites constituents in fenugreek genotypes after 5 days of visual symptoms appearance under field conditions.

Genotype	PDI	Total phenols	Total Free amino acids	Total soluble sugars
		mg^{-1} g FW		
AM-660	21.2	3.3	5.5	26.6
AM-661	18.3	2.5	5.6	25.0
AM-663	23.8	3.4	7.5	31.9
AM-651	40.0	1.8	2.4	41.4
AM-665	32.5	1.6	2.7	34.6
AM-232	8.8	4.4	2.1	20.9
AM-251	27.5	1.1	2.6	23.5
AM-86	26.3	1.3	1.1	19.8
AM-256	16.3	3.0	2.0	21.6
AM-286	31.3	1.9	1.6	24.6
AFg-1	21.3	3.3	1.3	29.8
S.Em.		0.14	0.15	1.2
C.D. at 5%		0.42	0.45	3.7

Where: PDI is percent disease index; FW is fresh weight

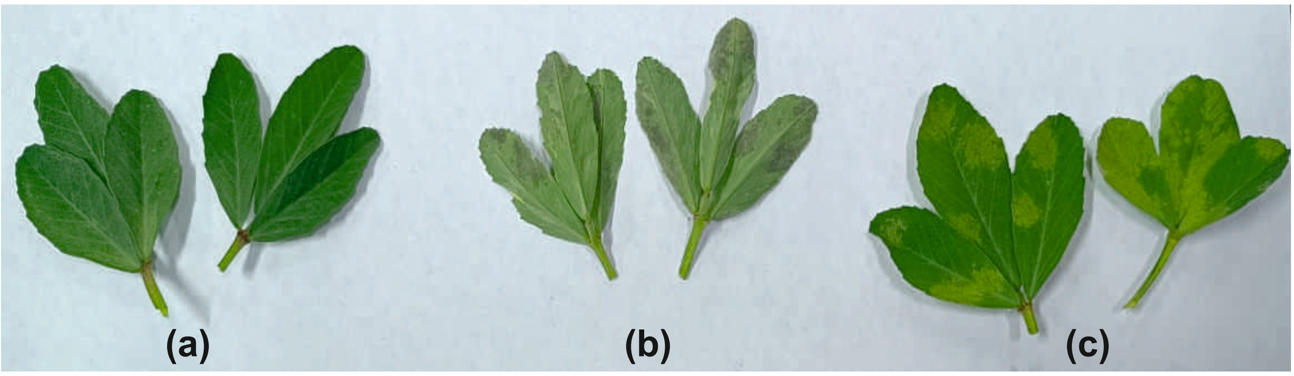


Fig 1: Downy mildew symptoms on lower and upper leaf surfaces. (a) Healthy leaves (b) fungal growth on lower leaf surface (c) Downy mildew symptoms on the upper leaf surface

Metabolite constituents

Total phenol content

Total phenol content was observed in the range of 1.1 to 4.0 mg g^{-1} FW in fenugreek genotypes. Genotypes with higher phenol content scored less PDI than those with lower phenol content. The results showed that higher phenol content imparted resistance to downy mildew disease in fenugreek. Higher phenol content was also observed in powdery mildew resistant genotypes of fenugreek (Avatar and Jatasra 2001). The accumulation of phenolic compounds in infected fenugreek leaves suggests their role in disease resistance.

Total free amino acids

The disease-induced changes in total free amino acids are given in Table 1. The genotype AM-253 (1.1 mg^{-1} g FW) possessed the lowest concentration of amino acid followed by AM-86 (1.3 mg^{-1} g FW) and AM-665 (1.6 mg^{-1} g FW) with a PDI of 27.5, 26.3 and 32.5 % respectively). While genotypes AM-232 and AM-661 with low PDI values (8.8 and 18.3 respectively) have 2.1 and 5.6 mg^{-1} g FW free amino acid content. These results showed no specific trend or correlation with resistance or susceptibility to downy mildew.

Total soluble sugars

The results presented in Table 1 showed that the concentration of total soluble sugars are varied in all genotypes. Genotypes AM-232 and AM-256 had lower soluble sugars (20.9 and 21.6 mg-1 g FW) and showed low PDI values (8.8 and 18.3 respectively) compared to genotypes that showed significantly higher sugars content (AM-651 and AM-665) and higher PDI (40.0 and 32.5). The higher amount of soluble sugar facilitates a faster rate of sugar transfer across the host-pathogen interface to enable sporulation and mycelium growth (Mahatma *et al.*, 2009).

Enzyme activities

Maximum polyphenol oxidase activity (Fig 2a) was observed in AM-256 (8.5 unit $min^{-1}g^{-1}$ protein) and minimum activity in AM-663 (6.1 unit $min^{-1}g^{-1}$ protein). Genotypes AM-256 and AM-661 had PDI scores of 16.3 and 18.3, respectively for downy mildew disease. The catalase activity was observed maximum in AM-660 (17.5μmol H_2O_2 min^{-1} g^{-1} protein) followed by AM-232 with a PDI score of 21.3 and 8.8 respectively (Fig 2b) . Minimum catalase activity (5.3μmol H_2O_2 min^{-1} g^{-1} protein) was observed in AM-286 with a PDI score of 31.3. While guaiacol proxidase activity (Fig 2c) was found maximum in AM-651 (1102.6μmol guaiacol min^{-1} g^{-1} protein) with a maximum PDI score (40) and minimum activity was observed in AM-256 (517μmol guaiacol min^{-1} g^{-1} protein). Thus higher activities of PPO and catalase enzymes were correlated with less disease incidence, while higher GPOX activity was correlated with susceptibility in the present study. In fenugreek genotypes, catalase enzyme may impart scavenging of hydroxen peroxide generated during downy mildew disease instead of POX enzyme. The increased peroxidase activity in susceptible genotypes suggests that the enzyme may be activated during the pathogenesis of downy mildew disease, rather than during disease resistance response. Similarly, higher POX activity was also observed in downy mildew susceptible genotypes of pearl millet than that of resistant genotypes (Mahatma *et al.*, 2011). The ratio of the increased activity of POX in leaves and roots of *Verticillium dahlia* susceptible tomato was also observed higher than that of resistant plants (Reuveni and Ferreira 1985).

Results of POX activity in the present study indicate that this enzyme is involved in the generation of H_2O_2 in plants ather than protection against the oxidative damage caused by reactive oxygen species (ROS). According to Gonzales and Rojas (1999), mobile extracellular peroxidase was found to be the primary source of reactive oxygen species, particularly H_2O_2, via the NADH with molecular oxygen. Lower catalase activity may compromise the plant's ability to scavenge ROS, leading to oxidative stress and increased disease susceptibility. After infection, the higher activity of PPO resulted in the formation of more toxic quinones and semiquinone radicals that may generate reactive oxygen species. These oxidative products are toxic to the extracellular enzymes produced by the pathogen (Swami *et al.*,2015). Reduced PPO activity might diminish the plant's capacity to produce antimicrobial compounds, while lower phenol content could weaken its antioxidant defense system.

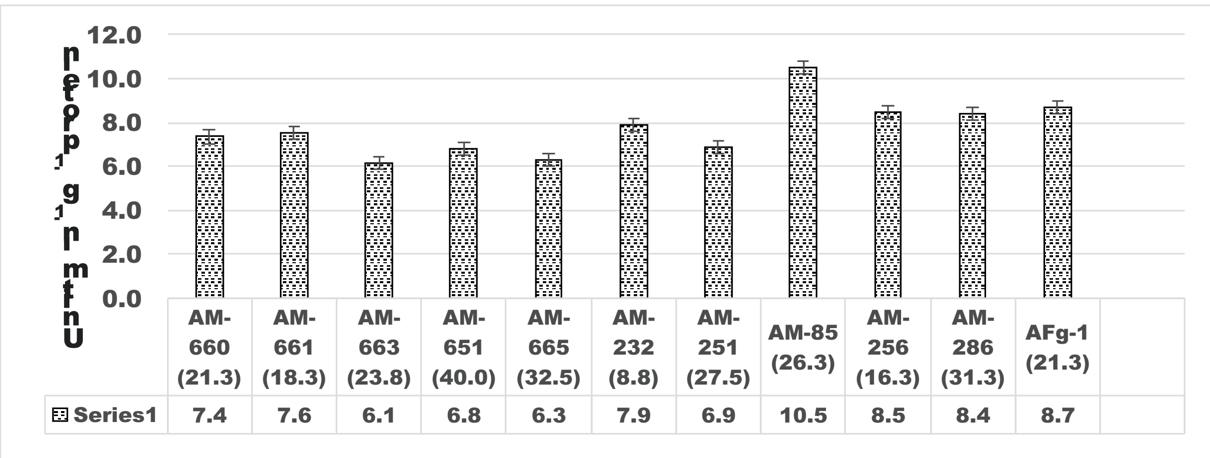
The PCA plot shows the relationship (Fig 3a) between different variables based on their contribution to two principal components (Dim1 and Dim2). These components show the most variance in the data. The Dim1 component explains 54.8% of the variance and component explains 27.4% of the variance. The variables are represented as vectors, and their direction indicate contribution to the principal components. PCA-biplot and correlation analysis (Fig 3b) revealed a significant positive correlation between PDI score and peroxidase activity while a significant negative correlation of PDI score was observed with catalase (-0.769**), PPO activity (-0.754) and phenol content (-0.570*). Similarly, More *et al.* (2021) observed higher PPO activity in immune genotypes of fenugreek against powdery mildew disease and a negative significant correlation (-0.766*) between PPO and PDI. The strong positive correlation between GPOX and PDI suggests that these two variables might be associated with disease conditions. Biochemical and PDI score data were further used to obtain hierarchical clustering (Fig 4) of genotypes which clustered fenugreek genotypes into three groups. The first group consisted of AM-232 and AM-256 with a PDI score of 8.8 and 16.3, respectively and identified as resistant sources for downy mildew disease of fenugreek. While in the opposite cluster, genotypes have higher PDI and grouped into susceptible genotypes. In conclusion, this study demonstrated that PPO, and catalase activity along with phenol content can distinguish the resistant germplasm.

Acknowledgement

The authors are grateful to the Director, ICAR-NRCSS, Ajmer for providing the facilities to conduct this experiment.

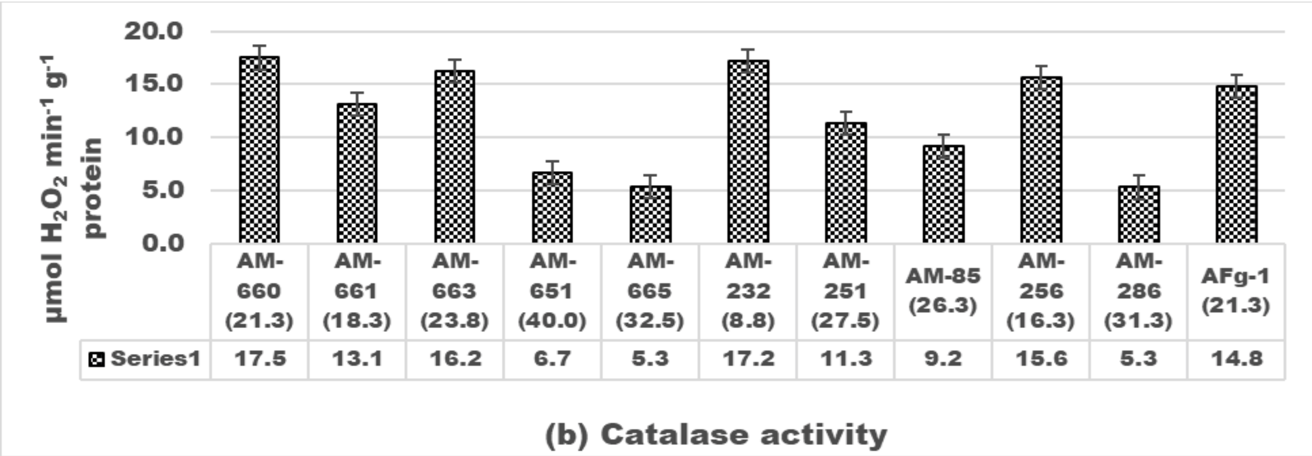
Conflict of interest

The authors declare no conflicts of interest.

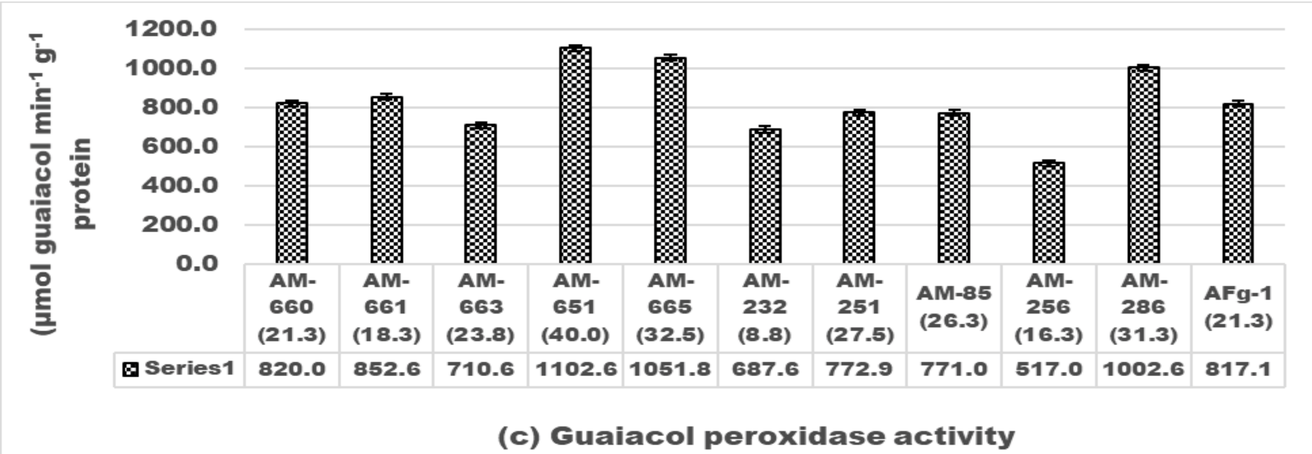


(a) Polyphenoloxidase activity

(a)



(b)



(c)

Fig 2: Enzyme activities in fenugreek genotypes after 5 days of visual symptoms appearance under field conditions. Value in parenthesis showing percent disease incidence of downy mildew in fenugreek. (a) Polyphenol activity (b) Catalase activity (c) Guaiacol peroxidase activity

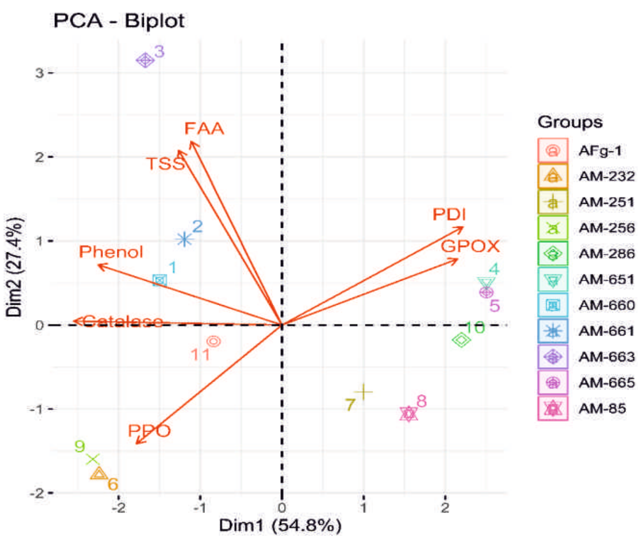


Fig. 3(a): PCA-Biplot analysis of fenugreek genotypes with biochemical parameters and PDI.

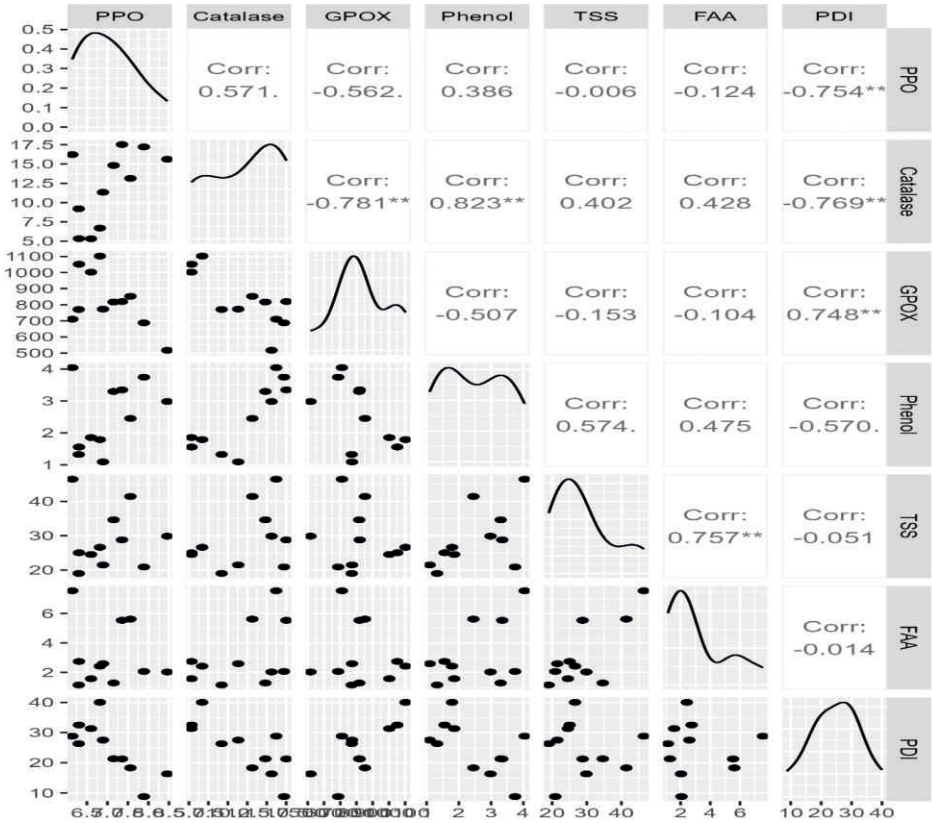


Fig. 3(b): Correlation analysis biochemical parameters with PDI during downy mildew disease in fenugreek

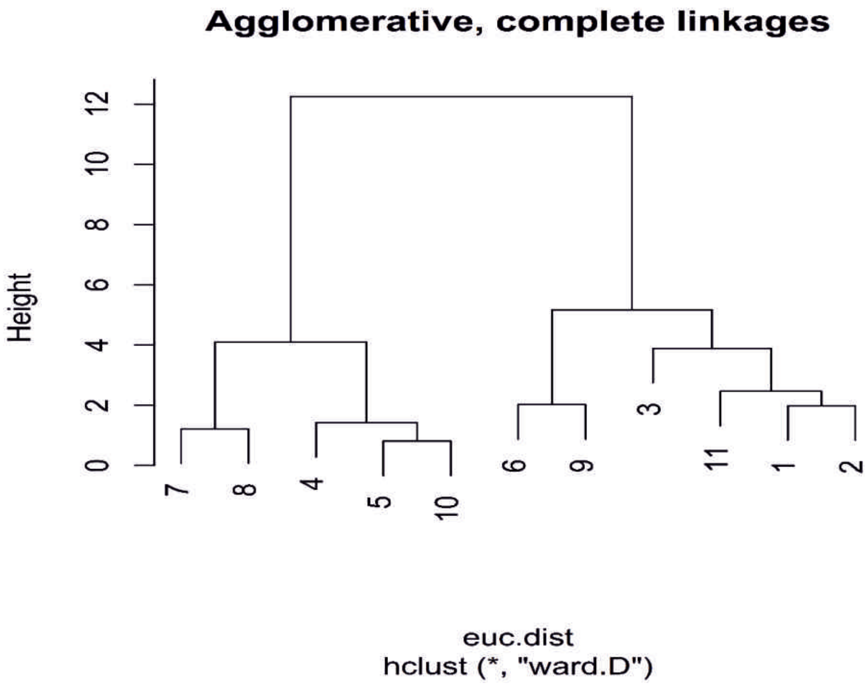


Fig 4: Dendrogram shows the hierarchical clustering of 11 fenugreek genotypes based on biochemical parameters and PDI. Where numbering in clusters denotes name of genotypes:1:AM-660, 2:AM-661, 3:AM-663, 4:AM-651, 5:AM-665, 6:AM-232, 7:AM-251, 8:AM-85, 9:AM-256,10:AM-286 and 11:AFg-1

References:

Avtar, R. and Jatasra, O.S. 2001. Rapid biochemical method for screening the fenugreek germplasm against powdery mildew disease. *Ind J Plant Gen Res.*, 14:390-91.

Franscistt, W.; David, F.B. and Robert, M.D. (1971). The estimation of the total soluble carbohydrate in cauliflower tissue. Experiment in plant phyisiology, Van, Nostrand. Reinhold Camp., New York, pp.16.

Gonzales, L.F. and Rojas, M.C. 1999. Role of wall peroxidases in oat growth inhibition by DIMBOA. *Phytochem.*, 50:931-937.

Goyal, S.K. and Sharma, P. 2012. Effect of environmental factors and inoculum age on fenugreek infection by *Peronospora trigonella* causing downy mildew. *Plant Dis Res.*, 27:149-152.

Hake, A.A., Jha, S., Jha, S. and Mahatma, M. 2018. Assesment of antioxidant and phenol related enzyme assays in Karanja (Derris indica). *Int J Chem Stud.*, 6(2):954-7.

Kapadia, C.V., Mahatma, M.K., Shrivastava, V., Ahmad, T. and Desai, R.T., 2013. Defense response of resistant and susceptible genotypes of castor (*Ricinus communis* L.) to wilt disease. *Arch Phytopath Plant Prot.*, 46(2):180-192.

Keen, N.T.; Ersek, T.; Long, M.; Bruegger, B. and Holliday, M. (1981). Inhibition of the hypersensitive reaction of soybean leaves to incompatible Pseudomonas spp. by blasticidin S, Streptomycin or elevated temperature. Physiol. Plant Pathol., 18 : 325-337.

Kumar, L., Mahatma, M.K., Kalariya, K.A., Bishi, S.K. and Mann, A. 2014. Plant phenolics: Important bio-weapon against pathogens and insect herbivores. *Popular Kheti*, 2(3):149-152.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folinphenol reagent. *J Biol Chem.*, 193:265.

Lee, Y.P. and Takahanshi, J. (1966). An improved determination of amino acid with use of ninhydrin. Anal. Biochem., 14 : 71.

Mahatma, M.K., Bhatnagar, R., Dhandhukia, P. and Thakkar, V.R. 2009. Variation in metabolites constituent in leaves of downy mildew resistant and susceptible genotypes of pearl millet. *Physiol Mol Biol Plants.*, 15(3):249-55.

Mahatma, M.K., Bhatnagar, R., Mittal, G.K. and Mahatma, L., 2011. Phenol metabolism in downy mildew resistant and susceptible genotypes of pearl millet. *Arch Phytopath Plant Prot.*, 44(7): 623-636.

Malik, C.P. and Singh, M.B. (1980). In : Plant Enzymology and Histo-Enzymology. Kalyani Publications, New Delhi.

More, N.D., Lohate, S.R. and Bhagat, A.A. 2021. Correlation of chemical and biochemical parameters with powdery mildew disease of fenugreek (*Trigonella foenum-graecum* L.). *Int J Chem Stud*, 9(1):29-33.

Reuveni, R. and Ferreira, J.F. 1985. The relationship between peroxidase activity and the resistance of tomatoes (*Lycopersicum esculentum*) to *Verticillium dahliae*. *J Phytopath.*, 112:193-197.

Swami, R.M., Mahatma, M.K., Parekh, M.J., Kalariya, K.A. and Mahatma, L. 2015. Alteration of metabolites and polyphenol oxidase activity in wilt resistant and susceptible pigeonpea genotypes during *Fusarium udum* infection. *Ind J Agric Biochem.,* 28(1):18-23.

Tör, M., Wood, T., Webb, A., Göl, D. and McDowell, J.M., 2023, October. Recent developments in plant-downy mildew interactions. In Seminars in Cell & Developmental Biol., Academic Press, 148:42-50..

Uppal, B.N., Patel, M.K. and Kamat, M.N. 1935. The Fungi of Bombay. *Bull Dept Agric.*, Bombay, pp176.