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Biochemical evaluation of green gram cultivars for resistance to *Cercospora* leaf spot disease

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Abstract

Green gram is highly valued for its rich nutritional profile, particularly its high protein content and essential micronutrients. It is a short-duration pulse crop. Its cultivation has expanded to several other regions due to its wide adaptability and significant nutritional value. The crop plays a significant role in improving soil fertility through biological nitrogen fixation. Despite its importance, the productivity of green gram remains relatively low, largely due to various biotic stresses. Among these, *Cercospora* leaf spot, caused by *Cercospora canescens*, is one of the most destructive diseases affecting the crop. First reported in India (Delhi), the disease is prevalent in humid tropical regions. Biochemical analysis of healthy and infected leaves of twenty-seven *Vigna radiata* genotypes showed that the resistant variety PDM 11 had the highest total phenols (8.17 mg/g), PO (10.69 unit-activity/min/g) and PPO (5.94 unit-activity/min/g) in diseased leaves. Infected leaves generally exhibited increased phenols, PO and PPO, while protein content decreased, though PDM 11 maintained the highest protein level (19.20 mg/g). Resistant genotypes consistently showed higher levels of these biochemical parameters than susceptible ones. These findings indicate that increased levels of phenols and defensive enzymes are associated with resistance, underscoring their potential role in breeding programs for disease-tolerant green gram varieties/genotypes.

Keywords: *Cercospora canescens*, Green gram, peroxidase, phenol, polyphenol oxidase, soluble protein

1. Introduction

Pulses can increase the productivity of cereal systems by improving soil health and fertility. (Masood *et al.*, 2002) [10]. The major pulse crops cultivated in India include chickpea, pigeon pea, green gram, and urdbean. Among these, green gram is the third most important pulse crop grown in the country. Green gram [*Vigna radiata* (L.) Wilczek] belongs to the family *Fabaceae* and subfamily *Papilionaceae*. It is a self-pollinated crop with a diploid chromosome number of $2n = 22$ (Karpechenko, 1925) [5]. Besides being an important source of nutrition for humans, green gram also improves soil fertility through its ability to fix atmospheric nitrogen (Muhammad *et al.*, 2004) [12]. Green gram is an important food legume widely consumed by humans because of its high nutritional value. It is a rich source of carbohydrates, proteins, vitamins and essential minerals, making it a vital component of balanced diets. Owing to its nutrient-dense composition, green gram plays a significant role in ensuring food and nutritional security, particularly in developing and resource-limited regions. The relatively low productivity of green gram can be ascribed to multiple constraints, of which biotic stresses represent a major limiting factor. Green gram is highly susceptible to several economically significant diseases, including *Cercospora* leaf spot (CLS), anthracnose, halo blight, powdery mildew, dry root rot, tan spot and yellow mosaic (Nair *et al.*, 2019) [14]. The incidence and severity of these diseases lead to considerable reductions in both yield and grain quality, thereby adversely affecting the overall productivity of the crop across different agro-climatic regions. *Cercospora* leaf spot (CLS) is a major fungal disease that significantly reduces green gram productivity. The fungus was first identified and designated as *Cercospora canescens*. Later, Chupp (1953) [3] documented its occurrence on green gram. Taxonomically, *Cercospora canescens* is classified within the class *Deuteromycetes*, order *Moniliales* and family *Dematiaceae*, reflecting its systematic placement among imperfect fungi.

Cercospora leaf spot was first reported in India, with its initial occurrence recorded in Delhi (Munjal *et al.*, 1960) [13]. This early report provided the first evidence of the disease's presence in the country. It is predominantly observed in the humid tropical regions of Malaysia, Bangladesh, Thailand, Indonesia and India, where its occurrence often leads to substantial yield losses and negatively impacts overall crop performance. *Cercospora* leaf spot initially appears on the leaves as small irregular to angular lesions with reddish-brown margins, a greyish centre and a yellow halo surrounding the spots. As the disease progresses, the lesions enlarge and may coalesce, causing severe damage to the foliage. The disease develops most rapidly at temperatures between 20-26 °C and under high relative humidity which are favourable for the growth and sporulation of the pathogen (Kumar *et al.*, 2011) [6]. It caused yield loss up to 47% in warm-wet season (Singh and Singh, 2014) [18].

2. Materials and Methods

2.1 Experimental site

The laboratory investigations were systematically performed at the Department of Biochemistry, SKN College of Agriculture, Jobner (Jaipur), under controlled conditions to ensure the precision, consistency and reliability of the results. Simultaneously, the field trials were conducted during the *Kharif* 2022 season at the Instructional Farm of Sri Karan Narendra Agriculture University, Jobner, Jaipur, Rajasthan, adhering to standardized agronomic practices.

2.2 Collection of samples

Leaf samples from 27 genotypes/varieties of green gram were collected in the morning from the experimental site for biochemical analysis. Both healthy and infected leaves were carefully selected and subjected to the estimation of phenol, peroxidase, polyphenol oxidase and protein content. All samples were systematically collected, meticulously handled to prevent contamination and placed in appropriately labeled paper bags to maintain sample integrity for laboratory

analysis. Standardized protocols were followed for the quantitative estimation of biochemical components

2.3 Biochemical changes in green gram induced by *Cercospora* leaf spot disease

The methods used for the estimation of different biochemical components are given below:

2.3.1 Estimation of phenol

Total phenol content was estimated using the Folin-Ciocalteu reagent method as described by Malick and Singh (1980) [9].

2.3.1.1 Reagents used for estimation

80% ethanol, Folin-Ciocalteu reagent, 20% Sodium carbonate, Standard Stock solution: 100 mg catechol was dissolved in 100 ml water and Working solution: 2 ml of stock diluted to 100 ml water.

2.3.1.2 Procedure for phenol estimation

A 0.5 g sample was weighed and ground in a pestle and mortar with 5 ml of 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 20 minutes and the supernatant was collected. The residue was re-extracted with 2.5 ml of 80% ethanol and centrifuged at 5,000 rpm for 10 minutes. The supernatants were pooled and evaporated to dryness using water bath. The residue was dissolved in 5 ml of distilled water and thoroughly mixed using a vortex mixer. From this solution, 0.2 ml was pipetted into a test tube and the volume was made up to 3 ml with distilled water. Then, 0.5 ml of Folin-Ciocalteu reagent was added. After three minutes, 2 ml of 20% sodium carbonate solution was added. The contents were mixed thoroughly and the tubes were placed in a boiling water bath for exactly one minute. After cooling, the absorbance was recorded at 650 nm against a blank. A standard curve was prepared using different concentrations of catechol and the amount of phenol present in the sample was calculated from the standard graph.

$$\text{Total phenol (mg/g)} = \text{Dilution factor} \times \text{graph factor} \times \text{optical density}$$

2.3.2 Estimation of peroxidase

Peroxidase activity was determined following the spectrophotometric method described by Costa (2002) [4], which is based on the ability of peroxidase to catalyze the oxidation of pyrogallol in the presence of hydrogen peroxide.

2.3.2.1 Reagents used for estimation

1. Sodium phosphate buffer (0.1 M, pH 7.0): Prepare 0.1 M solutions of monosodium phosphate and disodium phosphate separately. Mix 390 ml of monosodium phosphate solution with 610 ml of disodium phosphate solution to obtain a 0.1 M buffer at pH 7.0.
2. Pyrogallol (20 mM): Dissolve 0.63 g of pyrogallol in 250 ml of distilled water to prepare a 20 mM substrate solution.
3. Hydrogen peroxide (12.3 mM): Dilute 0.14 ml of 30% H₂O₂ to 100 ml with distilled water. The absorbance of

this solution should be 0.485 at 240 nm. Prepare the solution freshly before use.

2.3.2.2 Procedure for peroxidase estimation

All steps were performed under cold conditions. A known quantity (0.5 g) of leaf tissue was homogenized in 5 ml of 0.1 M phosphate buffer (pH 7.0) using a pre-chilled mortar and pestle. The homogenate was centrifuged at 15,000 rpm for 15 minutes at 4 °C. The resulting supernatant was collected and used as the enzyme source. The reaction mixture consisted of 2 ml of phosphate buffer, 50 µL of pyrogallol, 100 µL of enzyme extract and 30 µL of hydrogen peroxide. The increase in absorbance was measured at 420 nm using a UV spectrophotometer at 30-second intervals for 3 minutes. Peroxidase activity was calculated on a fresh weight basis and expressed as units per minute per gram of sample.

$$\text{Enzyme activity (unit-activity/min/g)} = \frac{\text{Change in absorbance} \times \text{dilution factor}}{\text{Weight of the sample} \times \text{dilution factor}}$$

2.3.3 Estimation of polyphenol oxidase (PPO)

Polyphenol oxidase (PPO) activity was determined according to the method described by Mayer *et al.* (1965)^[11]. The assay is based on the oxidation of catechol by PPO, resulting in the formation of quinones, which produce a measurable increase in absorbance.

2.3.2.3 Reagent used for estimation

1. Sodium phosphate buffer 0.1 M (pH 6.5): Prepare separate 0.1 M solutions of monosodium phosphate and disodium phosphate. Mix 685 ml of monosodium phosphate solution with 315 ml of disodium phosphate solution to obtain a 0.1 M buffer at pH 6.5.
2. Catechol 0.01 M: Dissolve 0.27 g of catechol in 250 ml of distilled water to prepare a 0.01 M catechol solution.

$$\text{Enzyme activity (unit – activity/min/g)} = \frac{\text{Change in absorbance} \times \text{dilution factor}}{\text{Weight of the sample} \times \text{time duration}}$$

2.3.4 Estimation of soluble protein

Soluble protein content was estimated by the method of Lowry *et al.* (1951)^[8].

2.3.4.1 Reagent used for estimation

1. **Sodium phosphate buffer (0.1 M, pH 7.0):** Prepared by dissolving appropriate quantities of monosodium phosphate and disodium phosphate in distilled water and adjusting the pH to 7.0.
2. **Solution A:** 2% (w/v) sodium carbonate (anhydrous) prepared in 0.1 N NaOH.
3. **Solution B:** 0.5% (w/v) copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) prepared in 1% (w/v) sodium potassium tartrate. This solution was prepared freshly.
4. **Solution C (Alkaline copper sulfate reagent):** Prepared immediately before use by mixing 50 ml of Solution A with 1 ml of Solution B.
5. **Folin-Ciocalteu Reagent (FCR):** Diluted to 1 N prior to use.
6. **Bovine Serum Albumin (BSA):** Used for preparation of standard protein curve.

2.3.4.2 Procedure for soluble protein estimation

Sample of leaves (0.5 g) was homogenized in 5 ml of 0.1 M sodium phosphate buffer (pH 7.0) using a mortar and pestle. The homogenate was centrifuged at 16,000 rpm for 20 minutes and the supernatant was collected for protein estimation. An aliquot of 0.1 ml of the supernatant was taken in a test tube and the volume was adjusted to 1 ml with distilled water. To this, 5 ml of freshly prepared Solution C was added and mixed thoroughly. The mixture was incubated at room temperature for 10 minutes. Subsequently, 0.5 ml of diluted (1 N) Folin-Ciocalteu reagent was added, mixed well and incubated in the dark at room temperature for 30 minutes for color development. The absorbance was measured at 660 nm using a UV-Visible spectrophotometer against a reagent blank. The soluble protein content was calculated from the standard curve prepared using different concentrations of bovine serum albumin and expressed as ppm on a fresh weight basis of leaf tissue.

3. Results and Discussion

3.1 Comparative analysis of total phenolic content in healthy and diseased leaves

The concentration of total phenols (mg/g) varied between healthy and diseased leaves. In general, the total phenol

2.3.2.4 Procedure for polyphenol oxidase estimation

The enzyme extract was prepared by homogenizing 0.5 g of fresh leaf tissue in 3 ml of 0.1 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 15,000 rpm for 15 minutes at 4 °C and the supernatant was collected and used as the enzyme source.

The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer, 200 µL of enzyme extract and 200 µL of 0.01 M catechol. The increase in absorbance was recorded at 495 nm using a UV spectrophotometer at 30-second intervals for 3 minutes.

Polyphenol oxidase activity was calculated based on the linear change in absorbance per minute and expressed as units per minute per gram of fresh weight of sample.

$$\text{Change in absorbance} \times \text{dilution factor} \\ \text{Weight of the sample} \times \text{time duration}$$

content was higher in infected leaves compared to healthy leaves (Table 1 and Fig. 1). In healthy leaves of green gram, the total phenol content ranged from 3.97 to 6.35 mg/g, whereas in diseased leaves it ranged from 4.54 to 8.17 mg/g. Among the healthy plants, the highest phenol content was recorded in the resistant variety PDM 11 (6.35 mg/g), followed by ML 515 (6.27 mg/g), while the lowest content was observed in the highly susceptible variety IPM 410-3 (3.97 mg/g). Following disease infection, an increase in total phenol content was observed in the leaves of all genotypes/varieties. The maximum phenol content was recorded in the resistant variety PDM 11 (8.17 mg/g), followed by ML 515 (8.09 mg/g), whereas the minimum content was found in the highly susceptible variety IPM 410-3 (4.54 mg/g). The results of the study indicate that phenol content was comparatively higher in resistant and moderately resistant genotypes/varieties and lower in moderately susceptible and susceptible genotypes/varieties. Parbat *et al.* (2018)^[15] reported total phenol content was maximum in resistant genotypes than in susceptible genotypes of groundnut infected by tikka disease. Similarly, Praveen *et al.* (2018)^[16] observed that resistant genotypes of green gram contained higher levels of total phenols than susceptible genotypes. Kumar *et al.* (2021)^[7] also noted an increase in total phenol content in all groundnut varieties infected with *Cercospora* leaf spot disease. Bijali *et al.* (2021)^[2] reported that biochemical analysis showed lower phenol contents in infected plants compared to uninoculated plants.

3.2 Comparative analysis of peroxidase (PO) activity in healthy and diseased leaves

Peroxidase (PO) activity was measured in healthy and diseased leaves and the activity was expressed as unit activity per minute per gram of sample (unit activity/min/g). The results showed that PO activity increased in infected leaves compared to healthy leaves (Table 1 and Fig. 2). In healthy leaves, PO activity ranged from 5.05 to 8.09 unit activity/min/g, whereas in diseased leaves it ranged from 5.95 to 10.69 unit activity/min/g. Among the healthy plants, the highest PO activity was recorded in the resistant variety PDM 11 (8.09 unit activity/min/g), followed by ML 515 (7.98 unit activity/min/g), while the lowest activity was observed in the highly susceptible variety IPM 410-3 (5.05 unit activity/min/g). After disease occurrence, PO activity increased in the leaves of all genotypes/varieties. The maximum activity was recorded in the resistant variety

PDM 11 (10.69 unit activity/min/g), followed by MH 318 (9.87 unit activity/min/g), whereas the minimum activity was observed in the highly susceptible variety IPM 410-3 (5.95 unit activity/min/g). Overall, the results indicated that PO activity was higher in resistant and moderately resistant genotypes and lower in moderately susceptible and susceptible genotypes. The present findings are parallel with those of Bharti *et al.* (2017) ^[1], who reported that peroxidase activity increased in both susceptible and resistant genotypes of green gram; however, the increase was more rapid and pronounced in the resistant genotypes compared to the susceptible ones.

3.3 Comparative analysis of polyphenol oxidase (PPO) activity in healthy and diseased leaves

The activity of the enzyme polyphenol oxidase (PPO) was measured in healthy and diseased leaves of green gram and expressed as units per minute per gram (unit activity/min/g). The results indicated that PPO activity increased in infected leaves compared to healthy leaves. Data presented in Table 1 and Fig. 3 showed that PPO activity ranged from 3.00 to 4.97 unit activity/min/g in healthy leaves and from 3.29 to 5.94 unit activity/min/g in diseased leaves. Among the healthy plants, the resistant variety PDM 11 exhibited the highest PPO activity (4.97 unit activity/min/g), followed closely by ML 515 (4.92 unit activity/min/g). In contrast, the highly susceptible variety IPM 410-3 showed the lowest enzyme activity (3.00 unit activity/min/g). After disease infection, PPO activity increased in the leaves of all genotypes/varieties. The highest activity was recorded in the resistant variety PDM 11 (5.94 unit activity/min/g), followed by ML 515 (5.81 unit activity/min/g), whereas the lowest activity was observed in the highly susceptible

variety IPM 410-3 (3.29 unit activity/min/g). Overall, these results indicate that PPO enzyme activity was higher in resistant and moderately resistant genotypes and lower in moderately susceptible and susceptible genotypes.

3.4 Comparative analysis of total protein content in healthy and diseased leaves

The concentration of total protein (mg/g) varied in both healthy and diseased leaves. Total protein content was higher in healthy leaves compared to diseased ones (Table 1 and Fig. 4). In healthy leaves of green gram, total protein concentration ranged from 16.37 to 20.23 mg/g, while in diseased leaves it ranged from 15.68 to 19.52 mg/g. The highest protein content in healthy leaves was observed in the resistant variety PDM 11 (20.23 mg/g), followed by ML 515 (20.01 mg/g), whereas the lowest protein content was recorded in the highly susceptible variety IPM 410-3 (16.37 mg/g). After disease infection, total protein content decreased in the leaves of all genotypes/varieties. The maximum protein content in diseased leaves was found in the resistant variety PDM 11 (19.52 mg/g), followed by ML 515 (19.20 mg/g), while the lowest content was recorded in the highly susceptible variety IPM 410-3 (15.68 mg/g). Overall, the results indicated that protein content was higher in resistant and moderately resistant genotypes/varieties and lower in moderately susceptible and susceptible genotypes/varieties. Praveen *et al.* (2018) ^[16] found that resistant genotypes of green gram had higher concentrations of total protein content than susceptible ones. Sharma *et al.* (2020) ^[17] found that total protein content decreased following infection by *Alternaria* blight in cluster bean, whereas total phenol content increased in diseased leaf samples of different varieties.

Table 1: Changes in total phenol, peroxidase, poly phenoloxidase and total protein content in healthy and diseased leaves of green gram

S. No.	Genotype/variety	Total phenol* (mg/g)		% Increase over healthy	Peroxidase* (unit-activity/min/g fresh wt)		% Increase over healthy	Polyphenoloxidase* (unit-activity/min/g fresh wt)		% Increase over healthy	Total protein* (mg/g)		% Decrease over healthy	PDI*
		H.	D.		H.	D.		H.	D.		H.	D.		
1	ML 515	6.27	8.09	22.49	7.98	9.78	18.40	4.92	5.81	15.31	20.01	19.20	4.04	5.04
2	MH 318	6.17	7.95	22.38	7.92	9.87	19.75	4.85	5.37	9.68	19.97	19.14	4.15	6.90
3	PDM 11	6.35	8.17	22.27	8.09	10.69	24.32	4.97	5.94	16.32	20.23	19.52	3.50	4.87
4	MH 421	5.89	7.39	20.29	7.49	9.69	22.70	3.65	4.30	15.11	19.67	19.05	3.15	13.40
5	RMG 1186	5.27	6.89	23.51	7.57	8.54	11.35	3.83	4.57	16.19	19.05	18.56	2.57	12.89
6	RMG 1191	5.95	7.45	20.13	7.83	9.93	21.14	3.54	4.93	28.19	18.88	17.88	5.29	15.37
7	RMG 975	4.17	5.27	20.87	6.41	7.80	17.82	3.50	4.82	27.38	18.23	17.45	4.27	22.40
8	RMG 1164	4.25	5.31	19.96	7.32	8.55	14.38	3.37	4.90	31.22	18.36	17.49	4.73	20.80
9	RMG 1193	4.52	5.56	18.70	6.49	7.95	18.36	3.41	4.78	28.66	17.97	17.20	4.28	25.77
10	RMG 1175	4.39	5.41	18.85	6.84	7.35	6.93	3.44	4.67	26.33	18.17	17.41	4.18	28.04
11	RMG 1181	4.27	5.32	19.73	6.18	8.04	23.13	3.35	4.79	30.06	17.89	17.29	3.35	22.79
12	RMG 1189	4.05	5.12	20.89	6.36	7.87	19.18	3.29	4.65	29.24	17.67	17.22	2.54	29.50
13	RMG 1192	4.35	5.43	19.88	7.12	8.40	15.23	3.28	4.84	32.23	18.06	17.40	3.65	24.66
14	RMG 1196	4.57	5.58	18.10	6.81	7.97	14.55	3.43	4.75	27.78	17.35	16.95	2.30	28.16
15	MSJ 118	4.74	5.78	17.99	6.87	8.10	15.18	3.19	4.20	24.04	17.78	17.02	4.27	22.18
16	ML 2459	4.24	5.35	20.74	6.15	7.85	21.65	3.21	4.64	30.81	18.03	17.42	3.38	25.60
17	IC 11443-1	4.37	5.49	20.40	6.47	7.51	13.84	3.15	4.23	25.53	17.99	17.06	5.16	29.22
18	PUSA 0891	4.51	5.53	18.44	6.41	7.45	13.95	3.20	4.92	34.95	17.56	17.00	3.18	28.19
19	PM 16-23	4.50	5.44	17.27	6.22	7.54	17.50	3.18	4.67	31.90	18.09	17.24	4.69	21.50
20	TARM 18	4.21	5.26	19.96	6.29	7.92	20.58	3.39	4.81	29.52	18.12	17.36	4.19	23.56
21	ML 682	4.43	5.47	19.01	6.32	8.02	21.19	3.43	3.92	12.50	17.87	17.23	3.58	29.30
22	BM 5	4.67	5.75	18.78	6.79	7.34	7.49	3.23	3.91	17.39	17.47	16.91	3.20	20.79
23	RMG 1194	4.04	4.72	14.40	6.52	7.25	10.06	3.12	3.65	14.52	17.06	16.85	1.23	33.20
24	RMG 492	4.01	4.82	16.80	6.13	7.01	12.55	3.17	3.91	18.92	17.19	16.43	4.42	45.20
25	IPM 02-3	4.14	4.78	13.38	5.97	6.02	5.98	3.05	3.98	23.36	16.98	16.29	4.06	34.89
26	MH 1142	4.21	4.71	10.61	6.24	7.20	13.33	3.10	3.88	20.10	16.88	16.20	4.02	35.76
27	IPM 410-3	3.97	4.54	12.55	5.05	5.95	15.12	3.00	3.29	8.80	16.37	15.68	4.21	53.80
	SEm±	0.24	0.27		0.29	0.32		0.21	0.24		0.55	0.53		0.65
	CD(p = 0.05)	0.70	0.78		0.85	0.90		0.60	0.69		1.57	1.51		1.87

H = Healthy, D = Diseased *Average of three replications% = percent

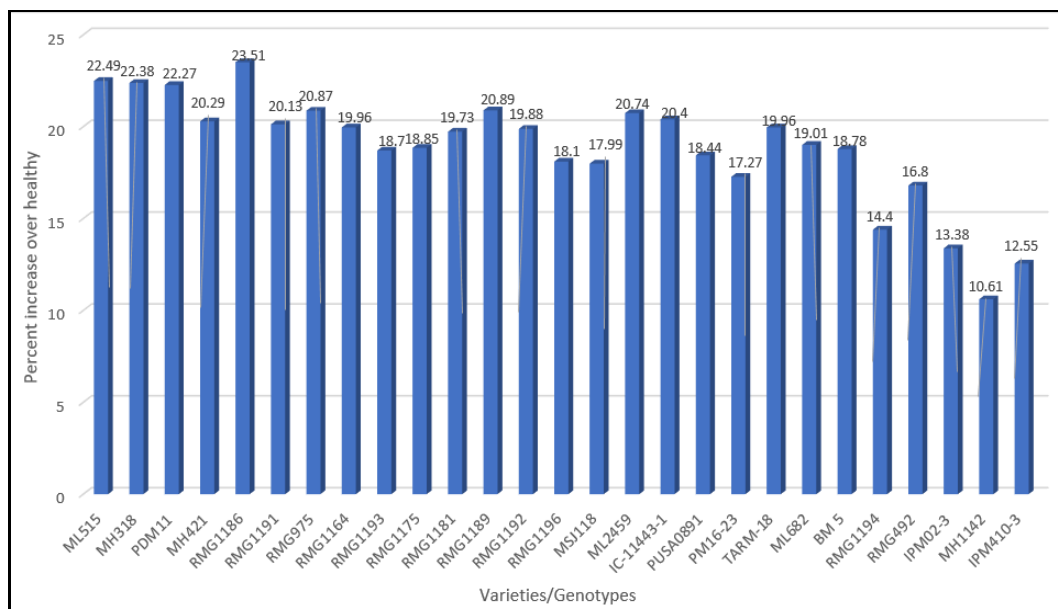


Fig 1: Changes in total phenol content in healthy and diseased leaves of green gram

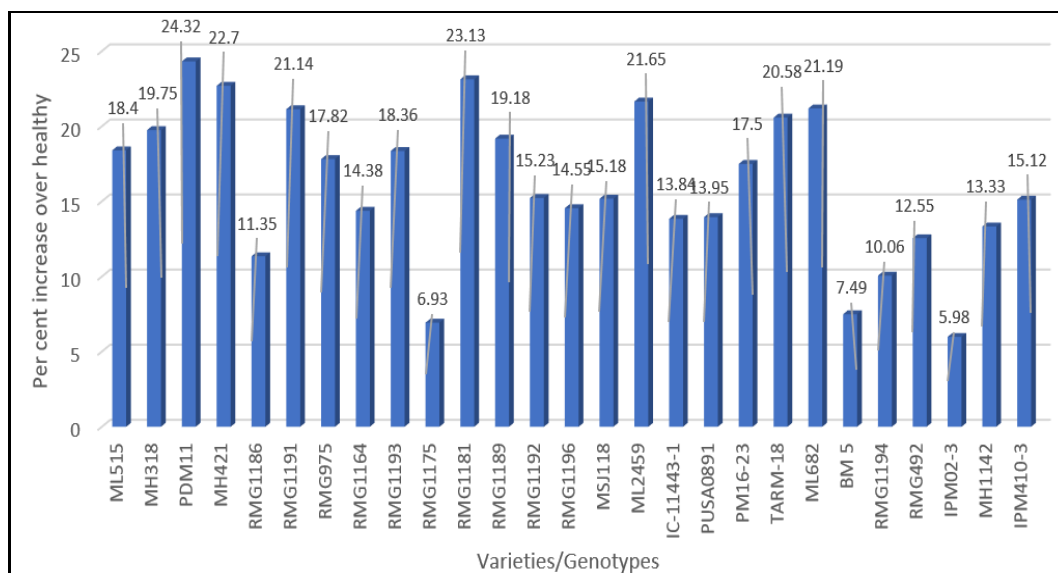


Fig 2: Changes in peroxidase enzyme activity in healthy and diseased leaves of green gram

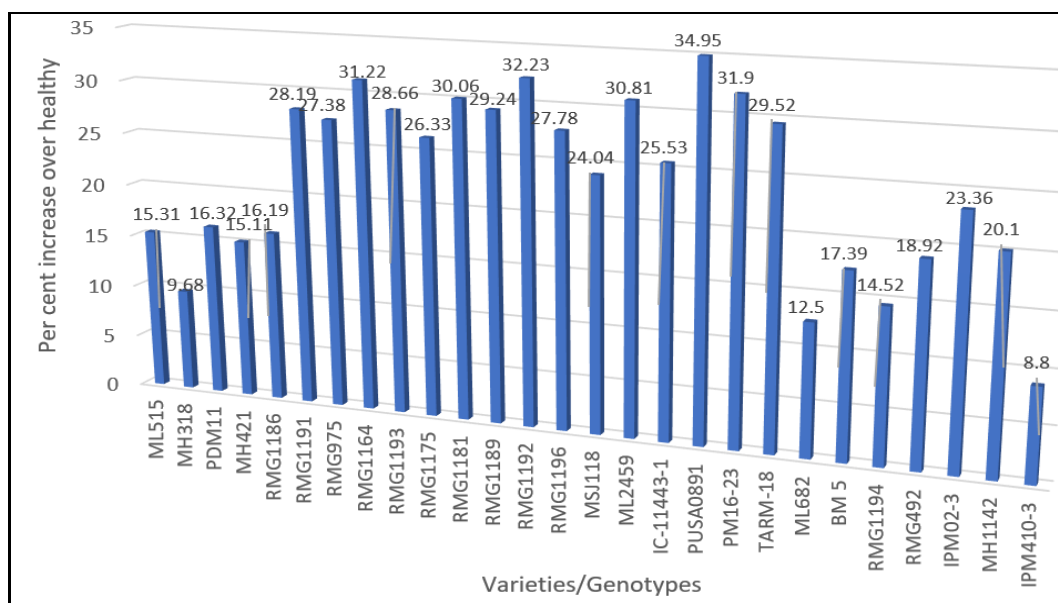


Fig 3: Changes in poly phenoloxidase enzyme activity in healthy and diseased leaves of green gram

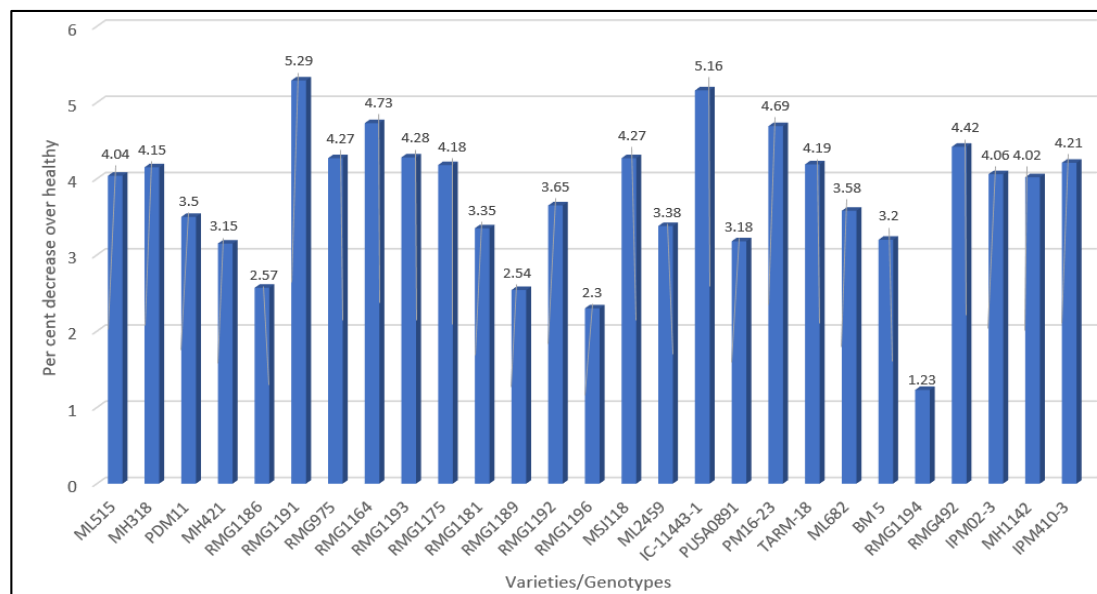


Fig 4: Changes in total protein content in healthy and diseased leaves of green gram

4. Conclusion

Green gram is an important pulse crop, but its productivity is significantly affected by *Cercospora* leaf spot caused by *Cercospora canescens*. The study revealed that disease infection increased total phenol content, peroxidase (PO) and polyphenol oxidase (PPO) activities, while reducing protein content. Among the tested varieties, PDM 11 showed the strongest biochemical response, while IPM 410-3 recorded the weakest. The results indicate that phenols, PO and PPO play an important role in disease resistance and may serve as useful biochemical markers for screening resistant green gram genotypes.

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6. Conflict of interest: None.

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