Pak. J. Bot., 58(1): 307-317, January 2026.

ARTICLE | DOI: http://dx.doi.org/10.30848/PJB2026-1(16)

ELUCIDATION OF PHYSIO-BIOCHEMICAL CHANGES IN MANGO PLANTS INCITED BY *XANTHOMONAS AXONOPODIS* PV *MANGIFERA INDICA*

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Abstract

Enzymatic substances in plants are the key compounds that help combat various biotic diseases; however, the interaction between these compounds and pathogens is a complex phenomenon. The balanced concentration of these biomolecules is crucial for regulating plant defense against the attack of invading pathogens. Therefore, in a contemporary study, biochemical profiling was performed to assess the alterations in biochemical substances of mango leaves induced by Xanthomonas axonopodis pv. mangifer*ae*. In this regard, six mango varieties (three resistant and three susceptible) were selected after two consecutive years of screening, and the pathogen was artificially applied using the syringe method. Leaves of resistant (Langra, White Chaunsa, Sensation) and susceptible (Anwar Ratol, Dusehri, Black Chaunsa) varieties from un-inoculated and inoculated groups were collected and analyzed for variations in biochemical substances and certain morphological attributes. Significant variations ($p \leq 0.05$) were observed in the biochemical compounds among the treatment types. Susceptible mango varieties exhibited 0.19, 387.17, 10.25, 3.30, 3.28 (µmol/mg), 13.55 and 16.5 mg/dry weight, resistant type expressed 0.24, 468.05, 15.74, 4.48, 3.49 (µmol/mg), 18.28 and 21.28 mg/dry weight of peroxidase, superoxidase dismutase, catalase, hydrogen peroxide, total soluble proteins, total phenolic contents and total soluble sugars respectively, while in case of groups, inoculated plants showed 0.13, 288.42, 3.15, 1.53, 2.85, 6.45 and 9.40 µg/g and un-inoculated group expressed 0.30, 566.78, 22.84, 6.25, 3.92, 25.39 and 28.39 µg/g. Photosynthetic rate, transpiration rate, stomatal conductance, and chlorophyll contents (total, a, and b) were observed to be high in healthy plants as compared to inoculated ones. At the same time, water use efficiency was observed in greater amounts in inoculated plants as compared to healthy plants. The outcome of this study can be used to develop biochemical markers to identify resistant mango sources against bacterial black spot disease.

Key words: Bacterial black spot; Biochemical profiling; Artificial inoculation; Biochemical compound and pathogen interaction; Nested structured design

Introduction

Mango (*Mangifera indica* L.) is a famous tropical fruit grown in several countries of the world. It belongs to the family Anacardiaceae and is the second most important fruit crop of Pakistan (Khan *et al*., 2010). It has an excellent taste, attractive aroma, and high nutritional value, containing sugar, acids, polyphenols, and antioxidants (Lebaka *et al*., 2021). Several biotic diseases, including mango malformation, powdery mildew, anthracnose, sudden death syndrome, and bacterial black spots, affect mango. Among these diseases, mango bacterial black spot (MBBS) poses a significant threat to mango-growing regions worldwide. The causal agent of MBBS disease is a rod-shaped, gram-negative bacterium known as *Xanthomonas axonopodis* pv. *mangifera indicae. (Xmi)*.

Disease occurrence in mango plants has a severe impact on their quality, resulting in premature fruit drop and substantial economic losses. In addition to reducing yield, *Xmi* negatively impacts fruit trade by altering the appearance of the fruit. Younger leaves are more susceptible to MBBS than mature ones, with lesions forming when bacteria come into direct contact with mesophyll cells after inoculation. Furthermore, symptom expression is influenced by environmental conditions. It produces small, water-soaked, raised, angular black spots with greasy margins, sometimes accompanied by a chlorotic halo (Cooke *et al*., 2009). During high humidity conditions, oozing of latex occurs from these lesions containing bacteria. Spots are usually tiny, but when these lesions coalesce, they form large necrotic areas on the mango leaf. After a few months of bacterial infection, spots on leaves become dry and turn brown to gray. Abscission of leaves occurs under severe attack by MBBS disease, and they become defoliated, eventually falling to the ground (Crane and Gazis, 2020).

Mango plants express various types of changes in biochemical compounds after the attack of bacterial pathogens (Singh *et al*., 2008). It causes up- or down-regulation of biochemical substances, but little research is available on the interaction between plant pathogens and biochemical compounds (Kaur *et al*., 2022). A reduction in biochemical substances in plants affects their physiological function, such as reduced nutrient uptake, absorption, assimilation, consumption, and mobility (Gomes *et al*., 2014). In diseased plant parts, the consumption and translocation of these enzymatic compounds are influenced by pathogens that cause variations in several biochemical compounds, which play a

Received: 11-02-2025 | Revised: 30-06-2025 | Accepted: 22-07-2025 | Online: 02-09-2025

crucial role in the occurrence of disease (Huber and Jones, 2013). The production of phenolic compounds enhances plant resistance and helps combat pathogen infection (Lattanzio *et al*., 2006). Plants activate their defense mechanism by producing various phenolic and sugar compounds that strengthen their cell walls and prevent the penetration of pathogens. It also reduces the cell wall's susceptibility to degrading enzymes and blocks pathogen toxins from entering the plant cell. (Boriboonkaset *et al*., 2013). Plants produce reactive oxygen species (ROS) in response to pathogen attack, which are toxic to both the pathogen and the plant. Resistant plants containing high SOD amount regulate the level of ROS by converting superoxide radicals into oxygen and hydrogen peroxide resulting in reducing the oxidative stress (Bailly, 2019). The production of ROS is influenced by a complex network of enzymes, including peroxidase, oxides, catalase, and superoxide dismutases (Hossain *et al*., 2009). SOD corresponding genes are upregulated, increasing the SOD level, which enhances host plant resistance against diseases caused by bacterial and fungal pathogens (Voloudakis *et al*., 2006). Determining physiological parameters such as photosynthesis, transpiration, stomatal conductance, and chlorophyll content is essential for evaluating plant health and performance, helping researchers understand how plants respond to biotic stresses. Water-use efficiency and leaf area can guide breeding programs aimed at developing resistant and high-yielding crops by selecting for traits that enhance plant performance under varying environmental conditions. Recognizing the significance of the issue, our study examined the biochemical compounds present in both healthy and inoculated mango plants (Usman *et al*., 2024). Identifying these compounds can help researchers develop biochemical markers to pinpoint resistant mango varieties. Additionally, these findings shed light on how mango plants resist or succumb to diseases, paving the way for more effective strategies to manage MBBS and other plant infections.

Material and Methods

Material collection: All chemicals and reagents used were of analytical grade. For instance, potassium dihydrogen phosphate (KH_2PO_4), silver nitrate, metaphosphoric acid, Hydrochloric acid, dinitrosalicylic acid, and Sodium Nitrite were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium iodide, dichloroindophenol, and trichloroacetic acid were purchased from Merck (Darmstadt, Germany). Potassium Chloride, Di-potassium hydrogen phosphate, and EDTA were purchased from Samchun. Aluminum chloride and Catechin were purchased from PubChem. Acetone, F-C reagent, and Sodium Hydroxide were purchased from Riedel-de Haen (Germany), Oxford Lab Chem (India), and Merck Millipore (USA), respectively.

Experimental setup: One-year-old mango plants of three resistant (Langra, White Chaunsa, Sensation) and three susceptible (Anwar Ratol, Dusehri, Blace Chaunsa) varieties were grown in plastic pots under greenhouse conditions. After two weeks of transplantation, when the plants were fully established, inoculum (1×10^6 CFU/mL in water) was applied early in the morning by using the spray method. After one week of critical observation, the disease began to appear, and samples were collected for further processing.

Preparation of a sample for biochemical analysis from leaves of inoculated and uninoculated mango plants: Leaf samples were collected from both groups, i.e., inoculated and uninoculated, from resistant and susceptible varieties. The samples were collected early in the morning from the top, middle, and bottom of the plant's canopy. Samples were washed with tap water to remove surface dust and cut into small pieces. These pieces were mixed to form a composite sample of each group. A leaf sample of 0.5 g was taken and ground in a pestle and mortar along with potassium phosphate buffer (pH 5). These samples were centrifuged (H-200 NR, Kokusan, Japan) at 12000 rpm for 5 minutes. The supernatant was taken only and stored at -20°C using sterilized bottles in a refrigerator (PEL, PRGD-145) for further biochemical analysis.

Estimation of total soluble proteins (TSP) from inoculated and uninoculated mango leaves: Total soluble proteins were measured by preparing an enzyme extract of leaves according to the Bradford method (Bonjoch & Tamayo, 2001). In potassium phosphate buffer (pH 5), an enzyme extract of 40 µL was prepared, vortexed, and then centrifuged. Bradford Reagent of 160 µL was added to this extract and was left for five minutes. A spectrophotometer was used for measuring the absorbance at 595 nm.

Determination of superoxide dismutase (SOD) from leaves of inoculated and un-inoculated mango plants: For determining superoxide dismutase (SOD) activity in mango leaves, a reaction mixture was prepared by mixing 100 µL of enzyme extract, 100 µL of NBT, 200 µL of Triton X-100, 200 µL of potassium phosphate buffer (pH 5), 200 µL of methionine, and 800 µL of distilled water. The mixture was placed under ultraviolet light for 15 minutes. After that, riboflavin was added at 100 µL, and the absorbance at a 560 nm wavelength was estimated using a spectrophotometer (Giannopolitis & Ries, 1977). The amount of superoxide dismutase was calculated as the quantity of enzyme needed to induce a 50% reduction in Nitroblue Tetrazolium activity.

Assessment of peroxidase (POD) from leaves of inoculated and uninoculated mango plants: Estimation of peroxidase (POD) from mango leaves was done by preparing a reaction mixture which contained potassium phosphate buffer 800 µL (pH: 05), enzyme extract 100 µL, guaiacol (20 mM), and 100 µL of 40 mM H_2O_2. The absorbance at 470 nm was measured using an atomic absorption spectrophotometer (Liu *et al*., 2007). Peroxidase was determined by the following formula:

$$\text{Peroxidase } (\mu g/g) = \frac{\text{A470 x 1000}}{43.6}$$

Assessment of catalase (CAT) from leaves of inoculated and uninoculated mango plants: Catalase (CAT) from mango leaves was determined by preparing a reaction mixture composed of 100 µL of enzyme extract and 100 µL of H2O2 (5.9 mM). Absorbance was measured at a wavelength of 240 nm using a spectrophotometer (Lin *et al*., 2009).Catalases was determined by the following formula:

$$\text{Catalase } (\mu g/g) = \frac{\text{A240 x 1000}}{43.6}$$

Determination of H_2O_2 concentration from leaves of inoculated and uninoculated mango plants: Samples of mango plants (inoculated and uninoculated) were collected and cut into small pieces. A 50 mg fresh leaf sample was weighed on an analytical weighing balance and ground in a Tri-chloroacetic acid buffer using a grinder. Then, the solution was centrifuged at 12,000 rpm for 15 minutes at 4°C. The filtered solution was treated with potassium phosphate at pH 7, followed by exposure to potassium iodide. The resulting mixture was incubated in a digital incubator (RTI-250, Robus) for a duration of 5 minutes, and its absorbance was measured at 390 nm using an Absorbance reader (800-TS, BioTek). The concentration of H_2O_2 was measured in µmol/g fresh weight (Velikova *et al*., 2000). The following formula was used to calculate the amount of H_2O_2;

$$\text{Hydrogen peroxide (µg/g)} = \frac{\text{Blank absorbance} - \text{Sample absorbance}}{\text{Blank absorbance}} \text{ x100}$$

Estimation of total soluble sugars (TSS) from inoculated and uninoculated mango leaves: The anthrone reagent ($C_{14}H_{10}O$) method was used for the quantification of total soluble sugars (Yemm and Wills, 1954). By using a boiling water bath, a 100 mg sample was hydrolyzed along with 5 mL of 2.5 N HCl for 3 hours and then left to cool at room temperature. Sodium carbonate (Na_2CO_3) was used to neutralize the solution until effervescence ceased. 100 mL volume was made and centrifuged (H-200 NR, Kokusan, Japan). A supernatant was obtained, and 0.5 µL, along with aliquots at a rate of 1 mL, was taken for further studies. Working standards of 0.00, 0.20, 0.40, 0.60, 0.80, and 1 mL were prepared to create specific standards, with zero acting as the blank. Distilled water was added to the tubes to increase the volume to more than 1 mL, followed by the addition of anthrone reagent to a final volume of 4 mL. A boiling water bath was used for heating the specific solution, and absorbance was determined using a spectrophotometer at 630 nm.

Quantification of total phenol contents (TPC) from leaves of inoculated and uninoculated mango plants: The amount of total phenols was estimated by preparing a 10 mL extraction mixture and adding it to 1 g of a grinded mango leaf sample. The prepared sample was centrifuged at 10,000 rpm for 10 minutes, and then 100 µL of the supernatant was taken and added to 200 µL of F-C reagent (10%). Then 800 µL Na_2Co_3 (700 mM) was added and left for one hour. A spectrophotometer was used to record absorbance at 765 nm.

$$TPC\ \left(\mu\frac{g}{mL}\right) = \frac{Sample\ Absorbance - Blank\ Absorbaance}{0.005} \times \frac{1}{1}$$

Assessment of morphological attributes: To check the effects of the MBBS pathogen on the physiological attributes of the mango plant, data were recorded for all respective parameters at approximately 10 am on the 14th day of inoculation. During this period (from transplantation to data recording), the plants were kept under strict observation in greenhouse conditions.

Chlorophyll contents: To determine chlorophyll contents, fresh mango leaves were collected, grinded, and extracted in acetone (80% solution). Centrifugation was done at 12,000 rpm for 5 minutes to purify the enzyme extract. The supernatant was separated, and the absorbance was taken by a spectrophotometer (Model) at 645, 663, and 480 nm (Iqbal *et al*., 2016; Haq *et al*., 2016). While the chlorophyll contents were determined by following the formulas:

$$\text{Total chlorophyll (mg/g)} = 20.20A_{663} + 8.02A_{645}$$
$$\text{Chlorophyll a (mg/g)} = 12.7A_{663} + 2.69A_{645}$$
$$\text{Chlorophyll b (mg/g)} = 22.9A_{645} + 4.68A_{663}$$

Leaf gas exchange parameters: Leaf gas exchange parameters, including net photosynthesis, transpiration rate, and stomatal conductance, were measured on three mature leaves per replication between 10:00 a.m. and 12:00 p.m. using the LCi-SD Ultra Compact Photosynthesis System (ADC Bio Scientific Ltd., Global House, Hoddesdon, UK). Intrinsic water-use efficiency, indicating the balance between carbon assimilation and water loss, was determined by calculating the ratio of net photosynthetic rate to stomatal conductance and expressed in µmol mol^{-1} (Ehleringer & Cerling, 1995). The measurements were conducted under controlled environmental conditions, including a daytime temperature of 34.6°C, a nighttime temperature of 27°C, a relative humidity of 78.9%, and a 12-hour photoperiod.

Data analysis: Changes in biochemical compounds were measured using a Nested Structured Design, and the data were statistically analyzed using the PROC MIXED procedure of the Statistical Analysis System (SAS).

Results

Inoculated and uninoculated mango plant leaves expressed significant variations in POD contents, at 0.13 and 0.30 µmol/mg, respectively, and accounted for 8.16% of the total variance. Resistant and susceptible plants also exhibited significant variations regarding POD percentage. Mango varieties displayed a specific response to POD contents, accounting for 2.89% of the total variance (Table 1). White chaunsa and Anwar ratol exhibited the maximum (0.26 µmol/mg) and minimum (0.17 µmol/mg) amount of POD, respectively (Fig. 1 and Table 1).

Significant alterations of Superoxide dismutase contents between inoculated (288.42 µmol/mg) and uninoculated (566.72 µmol/mg) mango plants were observed with a total variance of 7.82% at $p<0.05$. Susceptible (387.17 µmol/mg) and resistant mango plants (468.05 µmol/mg) also displayed significant variation with total variance of 91.38 as described in Fig. 1 and Table 1. Total variance of mango varieties towards SOD concentration was 0.74%.

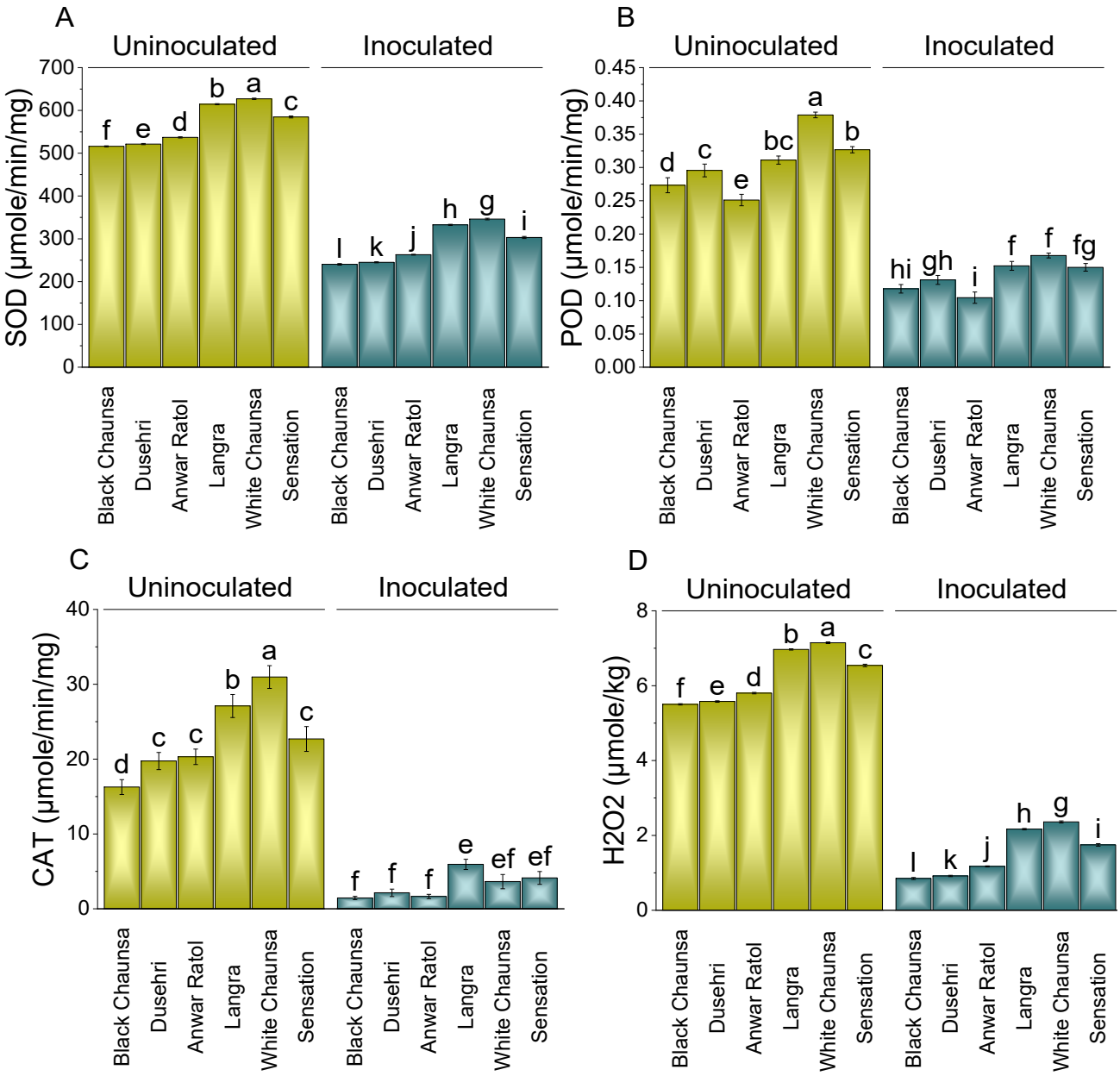

Fig. 1. Effect of inoculations on superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and hydrogen peroxidase (H2O2) of different mango varieties. The Fisher LSD revealed significant differences (p<0.05), with distinct letters indicating the means of three replicates and their standard error (SE).

Enzymatic variations in CAT contents were recorded between un-inoculated (22.84 µmol/mg) and inoculated (3.15 µmol/mg) mango plant leaves and accounted for 7.69 % of the total variance (Fig. 1 and Table 1). Resistant and susceptible plants also exhibited significant variations in CAT content percentage. Mango varieties displayed a significant response to CAT concentration, accounting for 2.21% of the total variance. White Chaunsa and Dusehri accumulated the maximum and minimum concentrations of CAT (17.3 and 8.85 µmol/mg), respectively.

Significant alterations in H_2O_2 contents between inoculated (1.53 µmol/kg) and uninoculated (6.25 µmol/kg) mango plant leaves were observed with total variance of 5.93% at p<0.05 as described in Tables 1 and 2. Susceptible (3.30 µmol/kg) and resistant mango leaves (4.48 µmol/kg) also displayed significant variation. The total variance of mango varieties in response to H_2O_2 concentration was 0.53%. White chaunsa and black chaunsa expressed maximum (4.75 µmol/kg) as well as minimum (3.17 µmol/kg) H_2O_2 concentration, respectively (Fig. 1 and Table 1).

Biochemical alterations were also observed in both inoculated and uninoculated mango plant leaves to estimate total soluble protein (TSP) content. Leaves of un-inoculated and inoculated mango plants expressed 3.92 µmol/mg and 2.85 µmol/mg TSP content, respectively, with a total variance of 3.78%. Similarly, the resistant and susceptible types exhibited 3.49 µmol/mg and 3.28 µmol/mg, respectively, with a total variance of 95.49%, as described in Fig. 2 and Table 1.

Total phenolic contents (TPC) were determined from un-inoculated and inoculated mango plant leaves, with mean values and percentage of total variance estimated through a Nested structured ANOVA. Mango leaves from

resistant and susceptible varieties demonstrated 18.28 mg/g and 13.55 mg/g TPC contents respectively with total variance of 87% while inoculated and un-inoculated mango plant leaves expressed TPC contents of amount 6.45 mg/g and 25.39 mg/g respectively with total variance of 5.57% as explained (Fig. 2 & Table 1).

Varieties Black Chaunsa, Dusehri, and Anwar Ratol with inoculated treatment caused a significant decrease in SOD (114.65%, 112.73%, and 104.20%), POD (132.07%, 125.43%, and 140.43%), CAT (1026.16%, 825.52%, and 1135.14%), and H_2O_2 (548.04%, 509.10%, and 395.35%) over the same varieties under uninoculated group. Varieties Langra, White Chaunsa, and Sensation with inoculated treatment showed a decrease in SOD (84.73%, 81.13%, and 92.84%) POD (104.38%, 125.83%, and 117.78%), CAT (356.74%, 752.29%, and 450.40%), and H_2O_2 (221.59%, 202.83%, and 274.73%) compared to the Langra, White Chaunsa, and Sensation varieties under uninoculated group (Fig. 1A, B, C, and D).

Table 1. Nested structured ANOVA for the assessment of peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), hydrogen peroxide (H2O2), total soluble proteins (TSP), total phenolic contents (TPC) and total soluble sugar (TSS) of inoculated and un-inoculated mango plant leaves.

Source of variance	DF	SS	MS	F value	P value	Variance components	Total variance %
Peroxidase (POD)							
Group (A)	2	0.07848293	0.0392	8.603	0.010*	0.001	8.16
Type (B)	1	0.7701	0.7701	19.638	0.047*	0.014	86.00
Variety (C)	8	0.0365	0.0046	9.797	0.000**	0.000	2.89
Error	96	0.0447	0.0005	-	-	0.000	2.96
Total	107	0.9297	-	-	-	0.016	-
Superoxide dismutase (SOD)							
Group (A)	2	176968.51	88484.25	32.286	0.000**	3175.690	7.82
Type (B)	1	2.092	2.09215	23.644	0.040*	37104.888	91.38
Variety (C)	8	21925.03	2740.62	118.124	0.000**	301.936	0.74
Error	96	2227.32	23.20	-	-	23.201	0.06
Total	107	2.293	-	-	-	40605.715	-
Catalase (CAT)							
Group (A)	2	1003.00	501.50	9.467	0.008*	16.612	7.69
Type (B)	1	10472.52	10472.52	20.882	0.045*	184.648	85.48
Variety (C)	8	423.80	52.97	5.304	0.000**	4.776	2.21
Error	96	958.90	9.98	-	-	9.989	4.62
Total	107	12858.22	-	-	-	216.025	-
Hydrogen peroxide (H_2O_2)							
Group (A)	2	38.09	19.0474	34.029	0.000**	0.685	5.93
Type (B)	1	601.80	601.8000	31.595	0.030*	10.792	93.49
Variety (C)	8	4.4780	0.5597	118.141	0.000**	0.062	0.53
Error	96	0.4548	0.0047	-	-	0.005	0.04
Total	107	644.83	-	-	-	11.543	-
Total soluble proteins (TSP)							
Group (A)	2	1.2477	0.6238	22.415	0.001*	0.022	3.78
Type (B)	1	30.7413	30.7413	49.277	0.020*	0.558	95.49
Variety (C)	8	0.2227	0.0278	20.798	0.000**	0.003	0.50
Error	96	0.128	0.0013	-	-	0.001	0.23
Total	107	32.34	-	-	-	0.584	-
Total phenolic content (TPC)							
Group (A)	2	703.53	351.7667	6.640	0.020*	11.066	5.57
Type (B)	1	9690.08	9690.0833	27.547	0.034*	172.932	87.00
Variety (C)	8	423.80	52.9751	5.304	0.000**	4.776	2.40
Error	96	958.90	9.9885	-	-	9.989	5.03
Total	107	11776.32	-	-	-	198.763	-
Total soluble sugar (TSS)							
Group (A)	2	721.0015	360.5007	6.805	0.019*	11.390	5.70
Type (B)	1	9739.5914	9739.5914	27.017	0.035*	173.687	86.98
Variety (C)	8	423.8007	52.9751	5.395	0.000**	4.795	2.40
Error	96	942.6397	9.8192	-	-	9.819	4.92
Total	107	11827.0334	-	-	-	199.691	-

*= Significant, **= Highly significant

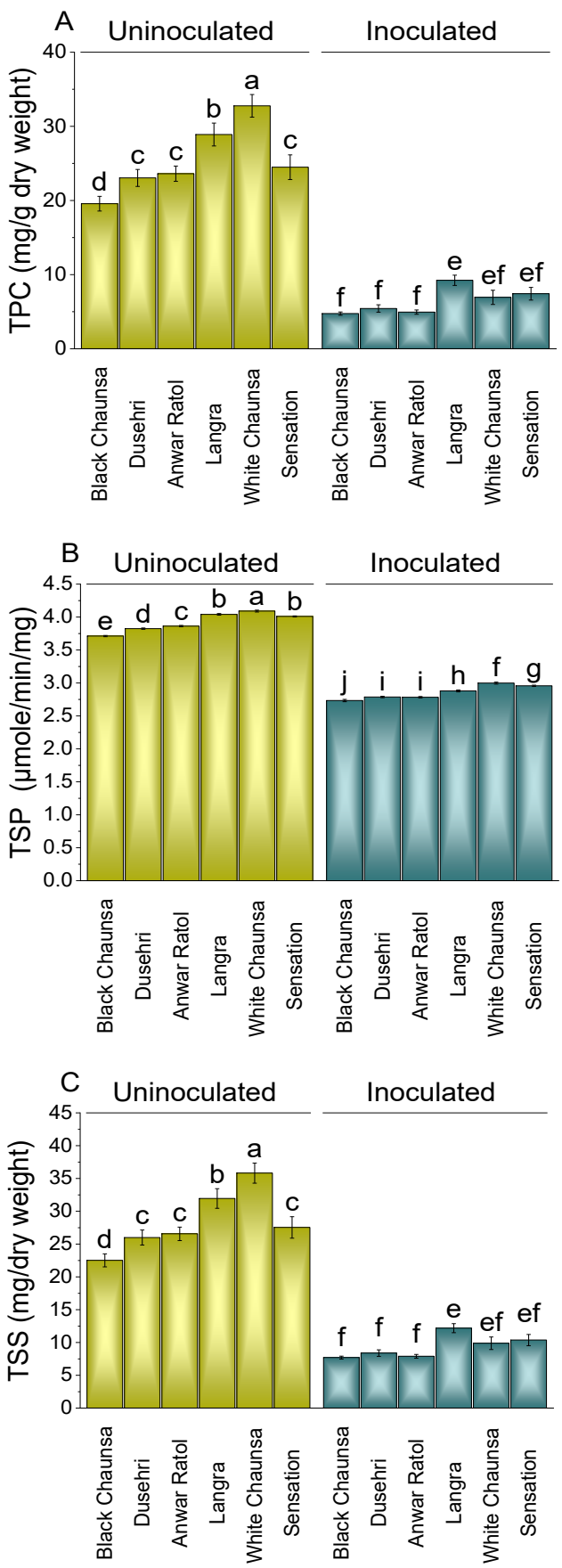

Fig. 2. Effect of inoculations on total phenolic content (TPC), total soluble proteins (TSP), and total soluble sugar (TSS) of different mango varieties. The Fisher LSD revealed significant differences (p<0.05), with distinct letters indicating the means of three replicates and their standard error (SE).

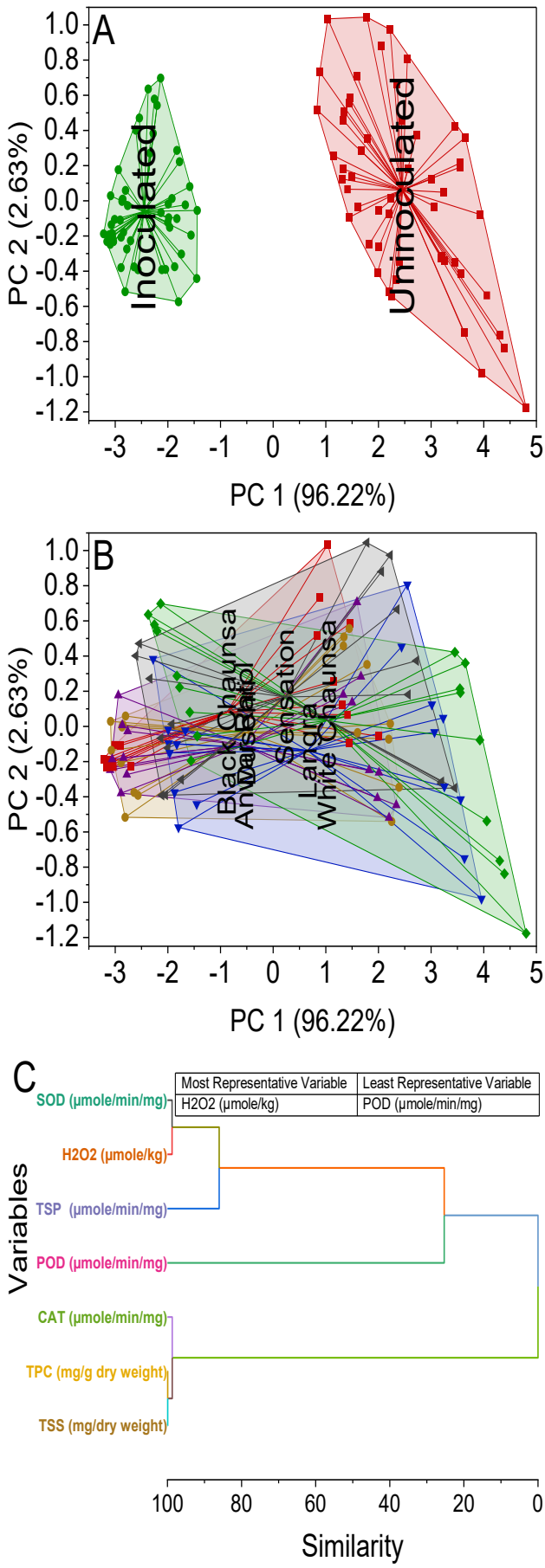

Fig. 3. Cluster plot convex hull for inoculation levels (TWBC) (A), different mango varieties (B), and hierarchical cluster plot (C) for studied attributes.

Among inoculated treatment varieties, Black Chaunsa, Dusehri, and Anwar Ratol demonstrate a decrease in TPC (312.41%, 324.13%, and 377.53%), TSP (35.79%, 37.21%, and 38.77%), and TSS (192.64%, 210.07%, and 236.45%) over the Black Chaunsa, Dusehri, and Anwar Ratol varieties under uninoculated treatment. Varieties Langra, White Chaunsa, and Sensation with inoculated treatment caused a decrease in TPC (213.00%, 372.60%, and 229.94%), TSP (40.34%, 36.45%, and 35.60%), and TSS (162.22%, 262.36%, and 165.47%) more than the Langra, White Chaunsa, and Sensation varieties in uninoculated treatment (Fig. 2A, B, and C).

The leaf gas exchange parameters, namely photosynthetic rate, transpiration rate, stomatal conductance, intrinsic water-use efficiency, and chlorophyll content, were significantly influenced by *Xmi* inoculation in the six mango genotypes (Table 2). Of the tested genotypes, the Langra variety proved superior, having the highest photosynthetic and transpiration rates, followed by the other varieties. The highest stomatal conductance was statistically recorded in the sensation variety, followed by Langra, white Chaunsa, and others. Among the six mango genotypes, white chaunsa had significantly higher total, "a", and "b" chlorophyll contents than other genotypes. In addition, the intrinsic water-use efficiency was recorded in the leaves of anwar ratol. *Xmi* inoculation tended to reduce the photosynthetic rate, transpiration rate, stomatal conductance, and leaf chlorophyll (total, "a", and "b") content in all resistance and susceptible genotypes statistically; however, water use efficiency expressed the reverse trend in response to bacterial inoculation. Following bacterial inoculation, the highest reduction in photosynthetic rate was observed in white chaunsa (20.51%), while the lowest reduction in photosynthetic rate was noted in anwar ratol (8.13%). Similarly, the highest reduction in transpiration rate was observed in langra (24.42%). The decrease in stomatal conductance in the tested genotypes following *Xmi* inoculation compared to the control ranged from 10.52 to 23.07%, being lowest in dohseri and highest in sensation. Similarly, maximum Chlorophyll (total, a, and b) contents were found in white chaunsa, which were 1.99, 1.44, 0.59 mg g^{-1} FW respectively, while minimum contents were found in black chaunsa, 0.79, 0.48, 0.27 mg g^{-1} FW, respectively. Collectively, *Xmi* inoculated plants expressed a lower rate of physiological attributes than that of un-inoculated ones. Pearson correlation heat map analysis among various enzymes of antioxidant defense system (A) and various physiological attributes (B) where SOD = Superoxide Dismutase; POD = Peroxidases; CAT = Catalases; H_2O_2 = Hydrogen Peroxide; TPC = Total Phenolic Contents; TSP = Total Soluble Proteins; TSS = Total Soluble Sugars.

The uninoculated group was distributed exclusively on the positive side of PC1, with score values ranging from 0.83 to 4.80. Notably, the highest PC1 scores were observed in samples such as 4.80636, 4.30502, and 3.95844, indicating greater variability within the uninoculated group. The spread along PC2 was moderate, ranging from approximately -0.98 to 1.04, indicating a slight dispersion vertically but strong horizontal clustering. In contrast, the inoculated group clustered tightly on the negative side of PC1, with score values ranging from -3.22 to -1.44. The PC2 scores for the inoculated group were more compact, ranging from -0.57 to 0.70, suggesting a more homogenous response to inoculation. The tight clustering and negative PC1 scores suggest a consistent shift in response due to inoculation across all mango varieties (Fig. 3A).

White Chaunsa showed the most dispersed pattern in the positive PC1 region, with scores ranging from 3.45 to 4.81 on PC1 and from -0.84 to 0.58 on PC2. Langra also clustered predominantly in the positive PC1 quadrant, with PC1 scores ranging from 2.55 to 3.96 and PC2 values from -0.98 to 0.80. Sensation was more centralized and compact, exhibiting scores from 1.74 to 3.45 on PC1 and -0.39 to 1.04 on PC2, indicating moderate variability. On the other hand, Black Chaunsa, Dusehri, and Anwar Ratol showed broader distributions across both positive and negative PC1 values, highlighting both intra- and inter-varietal variability. Notably, Black Chaunsa exhibited a wide PC1 range from -3.21 to 2.00, indicating high variability within this cultivar, while PC2 scores ranged from -0.23 to 1.03. Dusehri samples spanned across PC1 values from -3.09 to 2.39, with PC2 values ranging from -0.54 to 0.51. Similarly, Anwar Ratol spanned PC1 scores from -3.09 to 2.34, with PC2 ranging from -0.52 to 1.03 (Fig. 3B).

The strongest similarity (0.05694) was observed between total phenolic content (TPC) and total soluble sugars (TSS), indicating a close relationship in their response under the studied conditions. These were grouped early with high similarity, suggesting that both may be influenced by similar physiological or biochemical pathways. The next set of closely related variables included superoxide dismutase (SOD) and hydrogen peroxide (H_2O_2), which clustered together with a similarity value of 1.25044, highlighting their linked role in oxidative stress regulation. This pair further merged with catalase (CAT) and a composite cluster containing TSS and TPC at similarity scores of 1.29987 and 1.24293, respectively, reinforcing the close interaction among antioxidant enzymes and stress indicators. Further along the hierarchy, total soluble proteins (TSP) were grouped with the preceding clusters at a higher dissimilarity score of 13.9698, suggesting a relatively weaker but notable connection with the antioxidant defence system. Peroxidase (POD) showed the highest dissimilarity among the antioxidant enzymes, joining the cluster at a much later stage with a score of 74.69789, implying that it behaves more independently compared to other enzymatic antioxidants (Fig. 3C).

Superoxide dismutase (SOD) activity exhibited a nearly perfect correlation with hydrogen peroxide (H_2O_2) content (r = 0.9992), followed closely by total soluble proteins (TSP; r = 0.9894), total phenolic content (TPC; r = 0.9428), and total soluble sugars (TSS; r = 0.9436). Peroxidase (POD) activity also showed a strong positive correlation with SOD (r = 0.9534), catalase (CAT; r = 0.9190), and TPC (r = 0.9149). Similarly, CAT was highly correlated with TPC (r = 0.9991), TSS (r = 0.9992), and H_2O_2 (r = 0.9448). Notably, TPC and TSS showed an almost perfect correlation (r = 0.99996), indicating a strong interdependence between antioxidant content and sugar accumulation (Fig. 4).

Table 2. Impact of *Xanthomonas axonopodis* pv. *mangifera indica* inoculation on leaf gas exchange and chlorophyll contents of mango genotypes.

Treatment	Photosynthetic Rate (µmol m^{-2} s^{-1})	Transpiration Rate (mol m^{-2} s^{-1})	Stomatal Conductance (mol m^{-2} s^{-1})	Intrinsic water-use efficiency (µmol H_2O m^{-2} s^{-1})	Total Chlorophyll (mg g^{-1} FW)	Chlorophyll *a* (mg g^{-1} FW)	Chlorophyll *b* (mg g^{-1} FW)
Inoculated Resistant							
Langra	7.79 a	0.89 a	0.034 b	211.67 b	1.39 b	0.99 b	0.41 b
White Chaunsa	6.17 c	0.61 cd	0.029 c	186.51 c	1.61 a	1.06 a	0.45 a
Sensation	6.81 b	0.77 b	0.040 a	151.35 d	1.11 d	0.71 d	0.31 d
Inoculated Susceptible							
Anwar ratol	6.39 bc	0.65 c	0.029 c	221.98 a	1.09 d	0.81 c	0.33 cd
Dusehri	5.59 e	0.56 d	0.034 b	147.54 d	1.21 b	0.79 c	0.34 c
Black Chaunsa	5.89 d	0.49 e	0.028 c	189.12 c	0.79 e	0.48 e	0.27 e
Un-Inoculated Resistant							
Langra	8.33 a	1.11 a	0.040 b	191.35 a	1.69 b	1.19 b	0.45 b
White Chaunsa	7.11 c	0.83 c	0.039 b	161.53 c	1.99 a	1.44 a	0.59 a
Sensation	7.49 b	0.99 b	0.052 a	132.25 e	1.09 d	0.81 e	0.36 d
Un-Inoculated Susceptible							
Anwar ratol	6.91 d	0.76 d	0.034 c	181.51 b	1.31 c	0.91 d	0.41 c
Dusehri	6.21 e	0.68 e	0.038 b	139.56 d	1.35 c	0.99 c	0.43 bc
Black Chaunsa	7.41 b	0.65 e	0.039 b	159.35 c	0.99 e	0.71 f	0.35 d

Values in the column are mean of each variety and reaction type while lowercase letters were significantly different at $p \leq 0.05$ by Tukey's HSD test

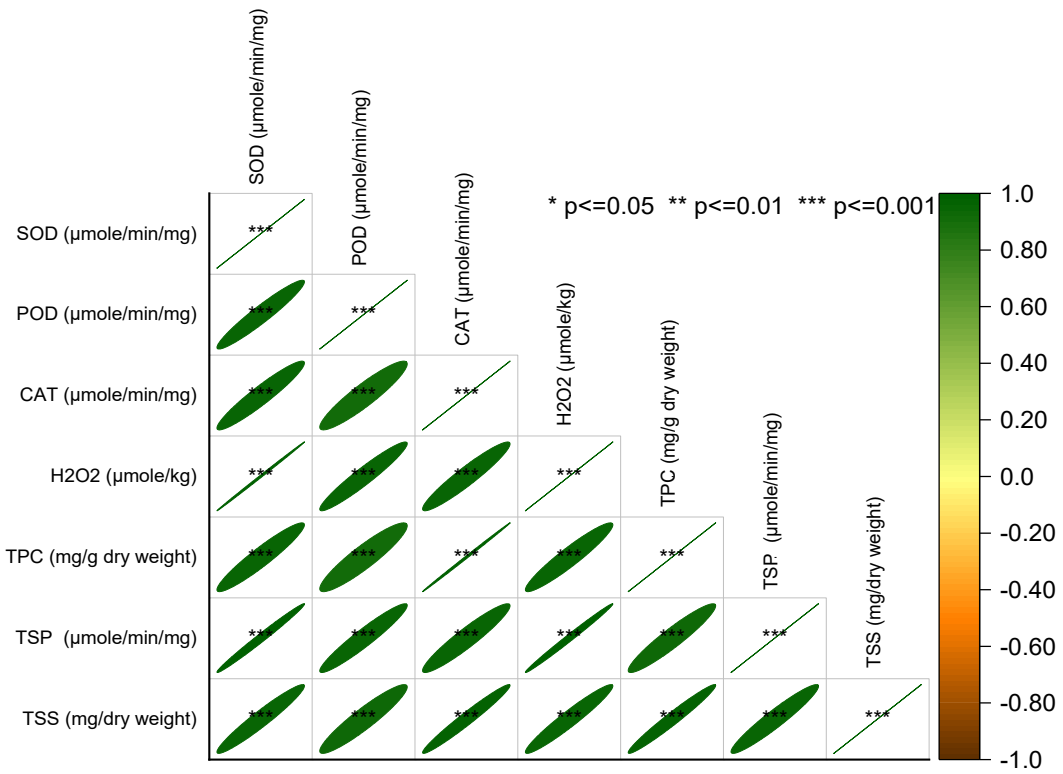

Fig. 4. Pearson correlation analysis for the studied attributes.

Discussion

Bacterial infection mediated enzymatic changes in plants causes up and down regulation of various phytochemicals which include peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), hydrogen peroxide (H_2O_2), total phenolic compounds (TPC), total soluble phenols (TSP) and total soluble sugars (TSS). Peroxidase (POD) plays a crucial role in the plant defense mechanism against invading bacterial pathogens. Xanthomonas infection triggers an increase in the production activity of peroxidases in resistant plants, which helps in reactive oxygen species (ROS) metabolism (Nowogórska and Patykowski, 2015; Mansoor *et al*., 2022). Reactive oxygen species are generated when Xanthomonas infects plants and cause oxidative stress to plant cells. Increasing POD production helps resistant plants detoxify and scavenge reactive oxygen species, resulting in reduced plant cell damage (Zaid and Wani, 2019). POD in resistant plants enhances the production of lignin compounds that strengthen the cell wall by polymerizing these substances, which ultimately increases the defense system against various bacterial pathogens (Hoffmann *et al*., 2020; Yuan *et al*., 2024). Peroxidases are also involved in the production of phenolic compounds that enhance plant resistance towards bacterial pathogens. Superoxide dismutase (SOD) is a crucial enzyme that plays a key role in defense mechanism of plants against bacterial pathogens. Plants produce reactive

oxygen species (ROS) as a result of pathogen attack, which are toxic to both the pathogen and the plant (Atiq *et al*., 2024). Resistant plants containing high SOD amount regulate the level of ROS by converting superoxide radicals into oxygen and hydrogen peroxide resulting in reducing the oxidative stress (Bailly, 2019). SOD in resistant plants also aids in inducing systemic acquired resistance (SAR), which triggers the signaling pathways resulting in the accumulation of pathogenesis-related proteins and the activation of defense genes that make the plant more resistant to bacterial pathogens (Tyagi *et al*., 2019). Catalase (CAT) is an essential antioxidant defense-related enzyme that breaks down hydrogen peroxide (H2O2), a reactive oxygen species (ROS) generated in plants during pathogenic infection. Resistant plants containing a high CAT level detoxify hydrogen peroxide into hydrogen and water, preventing cellular components from oxidative damage (Ighodaro and Akinloye, 2018). Bacterial pathogens also produce reactive oxygen species as an infection tool, and susceptible plants with low catalase levels are unable to neutralize the ROS effect, which increases the virulence of bacteria and makes plants more susceptible to infection.

When a pathogen attacks, plants prioritize the biosynthesis of defense-related substances while reducing other (e.g., growth-related) cellular activities (Usman *et al*., 2025). This permits a reduction in physiological parameters until pathogenic growth has been halted (Bolwell & Wojtaszek, 1997). This could benefit the plant by depriving biotrophic pathogens of essential nutrients. Various physiological parameters, such as chlorophyll contents (a, b, and total), photosynthetic rate, transpiration rate, stomatal conductance, and intrinsic water-use efficiency, were studied in six different genotypes (three resistant and three susceptible) of mango after artificial inoculation. The tested genotypes exhibited a reduction in their activities following X*mi* inoculation. However, the level of reduction was genotype-specific. Susceptible genotypes were found to be worse affected due to *Xmi* inoculation, as the highest reduction in total Chlorophyll, chl a, and chl b, stomatal conductance, transpiration rate, and photosynthetic activity were registered in these genotypes. The results of the current study align with those of Shad *et al*., (2023) and Guo *et al*., (2005), who found a significant reduction in chlorophyll content in infected plants with turnip mosaic virus. Probably, declining chlorophyll content per unit area of leaf, stomatal closure, and reduced stomatal closure upon infection are the reasons for a reduced photosynthetic rate (Sayed, 2003; Berova *et al*., 2007).

The photosynthetic rate in diseased plants is reduced due to the destruction of green leaf tissues and defoliation of leaves, leading to a decrease in photosynthetic area and ultimately reducing photosynthesis (Cheaib & Nabil, 2024). Similarly, various photosynthetic parameters, such as maximum photosynthetic rate, light saturation point, carboxylation efficiency, light compensation point, and dark respiration point, were higher in resistant mango varieties compared to susceptible mango varieties inoculated with bacterial pathogens (Hu *et al*., 2018). The rate of transpiration in infected mango plants was lower due to a reduction in maximal stomatal conductance compared to healthy plants. Bacterial pathogens affect stomatal behavior by altering hormonal signaling pathways, including abscisic acid (ABA), which regulates stomatal opening and closing (Hajji *et al*., 2009). Susceptible plants at the early stage of infection cause the opening of stomata due to the attack of bacterial pathogens, leading to higher transpiration rates. In contrast, resistant mango plants contain pathogens through a hypersensitive response (HR) and callose deposition, which prevent the further spread of bacterial pathogens and close their stomata as a defense mechanism, reducing the rate of transpiration (Shad *et al*., 2024; Jeyaraj *et al*., 2023). Bacterial pathogens in infected plants manipulate stomata to remain open, leading to increased stomatal conductance during the early stages of infection (Ahmad *et al*., 2024). Xanthomonas bacterium secretes effector proteins that suppress salicylic acid signaling and prevent closure of stomata (Atiq *et al*., 2023). During the later stages of infection, stomatal conductance decreased due to necrosis and damage to leaf tissues, which reduced the number of functional stomata (Zheng *et al*., 2010). Resistant plants exhibit controlled stomatal conductance in response to bacterial infection, and when the threat is neutralized, the plant reopens its stomata to restore normal photosynthesis and gas exchanges (Underwood *et al*., 2007). Water use efficiency (WUE) in inoculated plants was low due to production of toxins that damage plant cells and disrupt metabolic processes. Xanthomonas bacteria also secrete enzymes like cellulases and pectinases, which degrade plant cell walls, further reducing photosynthetic efficiency and WUE. Xanthomonas bacteria produce toxins and virulence factors that interfere with enzymes involved in synthesis of Chlorophyll. The infection triggers the production of reactive oxygen species (ROS) which degrade chlorophyll molecules and inhibit their synthesis (Silveira *et al*., 2015; Camejo *et al*., 2016). Xanthomonas infection also induces premature senescence (aging) in plant leaves, leading to the breakdown of Chlorophyll and other cellular components (Berna, 2022).

Conclusion

The results of a contemporary study reveal that the amount of all biochemical compounds is reduced in diseased mango leaves, while in healthy leaves, their concentration is high. The outcomes of current research work can serve as a baseline for researchers and breeders to develop biochemical markers for identifying disease-resistant sources against mango bacterial black spot disease.

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