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Original Research Paper

Defense-related responses in mango (*Mangifera indica* L.) cultivars 'Prior' and 'Kensington' differing in resistance to *Colletotrichum asianum*

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ABSTRACT

Mango is highly susceptible to anthracnose, a major postharvest disease caused by *Colletotrichum* spp. This study investigated the biochemical mechanisms underlying resistance in two contrasting cultivars, 'Prior' (moderately resistant) and 'Kensington' (susceptible), following inoculation with *Colletotrichum asianum*. 'Prior' exhibited significantly smaller lesion sizes compared to 'Kensington', indicating reduced disease severity. Higher activities of chitinase and β-1, 3-glucanase were observed in 'Prior', suggesting their critical role in degrading fungal cell walls and conferring resistance. In contrast, elevated polyphenol oxidase and peroxidase were observed in 'Kensington', potentially reflecting a weaker or delayed defense response. No significant differences were noted in phenylalanine ammonia lyase and superoxide dismutase activities between the cultivars. Two-way ANOVA revealed significant temporal effects for most enzymes, with genotype×time interactions for chitinase and β-1,3-glucanase. Correlation analysis showed co-regulation of these enzymes (r=0.70) and a negative association with PPO (r = -0.85). Principal component analysis (72.90% variance) clearly separated the two cultivars and reflected distinct temporal defense patterns. These findings demonstrate genotype-specific, time-dependent regulation of hydrolytic and oxidative defenses, providing a basis for breeding anthracnose-resistant mango cultivars.

Keywords: Anthracnose, *Colletotrichum asianum*, disease resistance, hydrolytic enzymes, mango, phenylpropanoid pathway

INTRODUCTION

Anthracnose, caused by *Colletotrichum gloeosporioides* species complex, is a major postharvest disease in mango, leading to yield losses ranging from 30% to 60%, and in severe cases, up to 100% under wet or humid conditions (Prakash et al., 1996). Recent multilocus phylogenetic analyses have identified *C. asianum* and *C. siamense* as the predominant species associated with mango anthracnose (Qin et al., 2019). The disease affects multiple plant parts, including leaves, flowers, young fruits, and twigs. In fruits, it manifests as irregular brown to black lesions often bearing pink to orange conidial masses, rendering them unmarketable. Resistance in unripe mango fruits is associated with preformed antifungal compounds such as 5-substituted resorcinols, gallotannins in the peel, and chitinase in the latex. However, the concentration of these compounds declines during ripening, increasing fruit susceptibility (Karunanayake et al., 2011).

Plants possess complex defense mechanisms composed of both constitutive and inducible components. One of the earliest responses to pathogen attack is the rapid accumulation of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (Torres, 2010). Enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase play key roles in modulating ROS levels and maintaining redox homeostasis, which is essential for effective plant defense (Niu et al., 2018). Phenylalanine ammonia lyase (PAL), a key enzyme in the phenylpropanoid pathway, is involved in the biosynthesis of lignin, phenolics, and phytoalexin that reinforce cell walls and hinder pathogen invasion (Pellegrini et al., 1994). POD is implicated in various cell wall associated defense processes, including lignification and suberization during pathogen attack (Lurie et al., 1997). Similarly, polyphenol oxidase (PPO) contributes to resistance by generating antimicrobial quinones through the oxidation of phenolics (Mohammadi & Kazemi, 2002).

Pathogenesis-related (PR) proteins are important defense molecules synthesized in response to biotic or abiotic stress. Among them, β-1,3-glucanases (PR-2) and chitinases (PR-3, PR-8, PR-11) are well-characterized for their role in fungal resistance. These enzymes break down essential structural components of fungal cell walls: β-1,3-glucans and chitin, respectively. Although present at low basal levels, they are significantly upregulated in response to pathogen attack (Salles et al., 2002).

Management of mango anthracnose through fungicides is increasingly challenged by resistance development and safety concerns related to chemical residues. Therefore, identifying resistant genotypes and their biochemical defense mechanisms is essential for sustainable disease management. In this context, the present study examines the biochemical responses of two contrasting mango cultivars 'Prior' (moderately resistant) and 'Kensington' (susceptible) following inoculation with *C. asianum*, a well-characterized and prevalent member of the *C. gloeosporioides* species complex. The study investigates key defense-related enzymes to elucidate their role in cultivar-specific resistance and provide insights for breeding anthracnose-resistant mango varieties.

MATERIALS AND METHODS

Plant material

Mature fruits of two mango cultivars, 'Prior' (moderately resistant) and 'Kensington' (susceptible), were obtained from ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru, India. Healthy, uniform fruits free from visible disease symptoms were harvested at physiological maturity and ripened under controlled laboratory conditions until the onset of ripening indicated by a change in peel color from the mature-green stage. Fruits were then washed with distilled water, surface-sterilized with 70% ethanol, air-dried and used for inoculation.

Pathogen culture and inoculation

C. asianum was isolated from anthracnose-infected mango fruits collected at ICAR-IIHR, Bengaluru, India. The isolate (IIHR_CA23) was cultured on potato dextrose agar and incubated at 25°C for 7 days. Molecular identification was confirmed by multilocus phylogenetic analysis of ITS, GAPDH, and ACT gene sequences (GenBank accession nos. PQ571852, PQ586487, and PQ570907) using reference sequences (Tovar-Pedraza et al., 2020). Mycelial plugs were used for inoculation because conidial suspensions tended to slide off the fruit and dry rapidly, resulting in inconsistent infection.

Early-ripening fruits were placed in ventilated plastic boxes (14×10.5×4.75 inches) lined with moist cotton to maintain high relative humidity (95–100%). Each fruit was wounded at 4–5 sites (≈1.5 mm deep) using a sterile needle and 5 mm diameter mycelial plugs of *C. asianum* were placed over the wounds. The needle was disinfected with 70% ethanol between fruits. Wounded fruits without mycelial plugs served as the non-inoculated controls. Both control and pathogen-inoculated fruits were incubated at 25±2°C under humid conditions (Tovar-Pedraza et al., 2020).

Sampling and storage

Peel tissue (approx. 2 mm thick) was excised from a 30 mm area around the inoculation site using a sterile scalpel. Pathogen-inoculated fruits were sampled at 1, 3, 5, and 7 days post-inoculation, whereas wounded, non-inoculated control fruits were sampled at 0 days. Multiple inoculation sites (4-5) within each fruit were treated as technical subsamples. For biochemical analyses, tissue excised from inoculation sites of three fruits was pooled to generate a single composite biological replicate, and three independent biological replicates were analyzed for each treatment and time point. Samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Enzyme extraction

Mango peel tissue (0.5 g) was homogenized in 5 mL of extraction buffer: 0.05 M potassium phosphate buffer (pH 7.0) for PAL, PPO, POX, and SOD assays and 0.05 M sodium acetate buffer (pH 5.0) for chitinase and β-1,3-glucanase. During extraction, 0.1% PVP and 0.1 mM EDTA were added to minimize phenolic interference. The homogenates were centrifuged at 12,000 × g for 15 minutes at 4°C and the supernatants were used for enzymatic assays.

Absorbance was measured using a Thermo Scientific™ Multiskan SkyHigh Microplate Spectrophotometer. Appropriate reagent blanks were included for all assays. For PPO and POX, absorbance was recorded at 1-min intervals, and reaction rates ($\Delta A\ min^{-1}$) were calculated from the linear phase of the reaction.

Chitinase assay

Chitinase activity was measured using colloidal chitin. The reaction mixture contained 150 µL of 1% colloidal chitin and 500 µL of enzyme extract, incubated at 40°C for 30 minutes (Ulhoa & Peberdy, 1992). The reaction was terminated by adding 300 µL of DNS reagent, followed by heating in a boiling water bath for 5 minutes. After centrifugation, the absorbance of the supernatant was measured at 550 nm to quantify reducing sugars (Miller et al., 1959). One unit of chitinase activity was defined as the amount of enzyme required to release 1 µg of glucose equivalents per minute per gram of fresh weight.

β-1,3-glucanase assay

β-1,3-glucanase activity was determined using laminarin (Sigma-Aldrich) and the dinitro salicylic acid method (Pan et al., 1991). The reaction mixture consisted of equal volumes (62 µL each) of 4% laminarin solution and enzyme extract, incubated at 40°C for 10 minutes. The reaction was terminated by adding 375 µL of DNS reagent, followed by heating in a boiling water bath for 5 minutes. The absorbance of the resulting solution was measured at 550 nm. One unit was defined as the release of 1 µg of glucose per minute per gram of fresh weight.

Phenylalanine ammonia lyase (PAL)

PAL activity was assayed as described by Dickerson et al. (1984). The reaction mixture (200 µL) contained 45 µL of enzyme extract, 55 µL of 25 mM Tris-HCl buffer (pH 8.8) and 100 µL of 12 mM L-phenylalanine. The mixture was incubated at 30°C for 2 h and the formation of trans-cinnamic acid was measured at 290 nm. Enzyme activity was expressed as nanomoles of trans-cinnamic acid produced per minute per gram of fresh weight.

Polyphenol oxidase (PPO)

PPO activity was assayed according to Selvaraj & Kumar (1995). The 200 µL reaction mixture contained 50 µL of crude enzyme extract, 144 µL of 0.05 M potassium phosphate buffer and 6 µL of 1.25% pyrogallol. Absorbance was measured at 450 nm for 6 min at 1 min interval, and absorbance changes were blank-corrected before activity calculation. Enzymatic activity was recorded as a change in absorbance per min per gram of FW.

Peroxidase (POD)

POD activity was assayed as described by Subhas (1990). The total reaction volume was 200 µL, containing 13 µL of 1% o-phenylenediamine, 13 µL of 0.3% hydrogen peroxide, 124 µL of 0.05 M potassium phosphate buffer and 50 µL of enzyme extract. Absorbance was measured at 450 nm for 6 min at 1 min intervals, and absorbance changes were blank-corrected prior to activity calculation. POD activity was expressed as the change in absorbance per minute per gram of fresh weight.

Superoxide dismutase (SOD)

SOD activity was determined following Du & Bramlage (1994). The reaction mixture (200 µL) contained 39 mM methionine (67 µL), 450 µM nitro blue tetrazolium (NBT; 33 µL), 12 µM riboflavin (33 µL), 10 mM EDTA (2 µL), 50 mM potassium phosphate buffer (pH 7.8; 5 µL), and enzyme extract (60 µL). Two identical sets were prepared; one was exposed to fluorescent light for 30 min, while the other was kept in darkness. Absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of NBT photoreduction. Enzyme activity was expressed as units per gram fresh weight.

Statistical analysis and visualization

Statistical analyses were performed in Python v3.10 using pandas, NumPy and statsmodels (McKinney, 2010). Lesion diameter data were analysed using independent-samples Student's *t*-test to compare the two cultivars at each time point. For each enzyme, two-way ANOVA was performed to evaluate the effects of genotype, time and their interaction. Genotype (Prior & Kensington) and time (0, 1, 3, 5 and 7 dpi) were treated as fixed factors, with three biological replicates per genotype×time combination. When significant effects were detected, means were separated using Duncan's multiple range test ($p<0.05$) in SPSS 16; all model fitting and assumption checks were conducted using replicate-level data in Python.

Multivariate analyses were performed using genotype × time treatment means from three biological replicates. Enzyme activities were Z-score standardized before multivariate analyses. Relationships among enzymes were examined using Pearson correlation analysis and visualized as a correlation matrix. Principal component analysis

Table 1 : Enzymatic activity of Chitinase and β-1, 3-Glucanase in Prior and Kensington genotypes in response to *C. asianum*

Time interval (dpi)	Chitinase (g^{-1} FW)*		β-1, 3-glucanase (g^{-1} FW)	
	Prior	Kensington	Prior	Kensington
0	$110.13^{b, p}$	$50.94^{b, q}$	$242.08^{d, p}$	$193.04^{d, q}$
1	$126.24^{a,p}$	$55.27^{a, q}$	$281.79^{c, p}$	$271.54^{a, p}$
3	$123.17^{a, p}$	$40.01^{d, q}$	$289.03^{bc, p}$	$228.12^{c, q}$
5	$90.11^{c, p}$	$43.87^{c, q}$	$305.68^{ab, p}$	$174.35^{e, q}$
7	$119.64^{a, p}$	$44.58^{c, q}$	$319.84^{d, p}$	$249.56^{b, q}$
SE(m)±	1.748		2.906	
CD	5.083		8.434	

1 Unit: µg glucose produced min^{-1} g^{-1} FW. *Data are presented as the mean of three replications (n=3). Values followed by the same lowercase letters (a–e) within the same column do not differ significantly among time intervals according to Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$. Values followed by the same lowercase letters (p, q) within the same row do not differ significantly between mango genotypes at the same time point ($p \leq 0.05$)

(PCA) was performed using the scikit-learn library. PCA scores were visualized using seaborn.scatterplot, with genotype and time represented by different colors and symbols.

RESULTS AND DISCUSSION

Disease resistance in 'Prior' and 'Kensington' fruits inoculated with *C. asianum*

Lesion diameter increased progressively over time in both mango genotypes following inoculation with *C. asianum*. At 5 days post inoculation (dpi), the moderately resistant genotype Prior developed significantly smaller lesions (0.68±0.07 cm) compared with the susceptible genotype Kensington (1.43±0.26 cm) (p=0.002). A similar trend was observed at 7 dpi, where lesion diameter in Prior (1.02±0.09 cm) remained significantly lower than that in Kensington (2.30±0.32 cm) (p=0.0005; Fig. 1).

Defense-related enzyme activities

Chitinase activity varied significantly over time after inoculation in both cultivars. Chitinase activity was significantly higher in Prior than in Kensington at all time points and was approximately two-fold higher (Fig. 2). Significant differences ($P<0.05$) in chitinase activity were observed between the two cultivars at each time point (Table 1).

Table 2 : Evaluation of PAL, PPO, POD and SOD activities in Prior and Kensington mango genotypes inoculated with *C. asianum*

Time interval (dpi)	PAL (ηmol trans-cinnamic acid min^{-1} g^{-1} FW)*		PPO (min^{-1} g^{-1} FW)		POD (min^{-1} g^{-1} FW)		SOD (g^{-1} FW)	
	Prior	Kensington	Prior	Kensington	Prior	Kensington	Prior	Kensington
0	$2.58^{bc, p}$	$2.51^{ab, p}$	$43.05^{d, p}$	$72.05^{c, q}$	$1.21^{b, p}$	$1.16^{c, p}$	$302.36^{ab, p}$	$295.56^{a, p}$
1	$2.71^{a, p}$	$2.61^{a, p}$	$55.00^{a, p}$	$91.69^{a, q}$	$1.51^{a, p}$	$1.80^{a, p}$	$332.23^{ab, p}$	$324.86^{a, p}$
3	$2.51^{cd, p}$	$2.44^{b, p}$	$46.36^{c, p}$	$83.27^{b, q}$	$0.96^{c, q}$	$1.42^{bc, p}$	$360.65^{a, p}$	$357.62^{a, p}$
5	$2.42^{d, p}$	$2.37^{b, p}$	$52.23^{b, p}$	$70.06^{c, q}$	$0.77^{d, q}$	$1.59^{ab, p}$	$293.48^{b, p}$	$280.34^{b, p}$
7	$2.66^{ab, p}$	$2.46^{b, q}$	$52.10^{b, p}$	$96.77^{a, q}$	$1.42^{a, q}$	$1.57^{ab, p}$	$290.25^{b, p}$	$274.23^{b, p}$
SE(m)±	0.025		1.613		0.072		3.141	
CD	0.073		4.682		0.208		9.405	

I Unit for PPO and POX: Change in absorbance per min per g of FW; 1 Unit for SOD: 50% inhibition of the NBT photoreduction rate. * Data are presented as the mean of three replications (n = 3). Values followed by the same lowercase letters (a–d) within the same column do not differ significantly among time intervals according to DMRT at $p \leq 0.05$. Values followed by the same lowercase letters (p, q) within the same row do not differ significantly between genotypes at the same time point ($p \leq 0.05$)

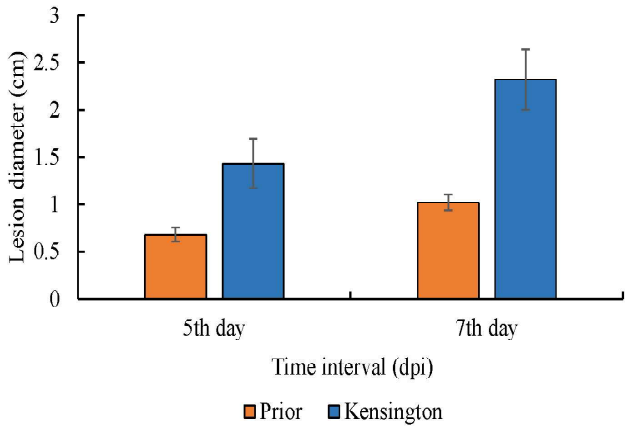

Fig. 1 : Disease resistance in Prior (MR) and Kensington (S) mango genotypes inoculated with *C. asianum*

β-1,3-glucanase activity was approximately 1.03–1.75-fold higher in Prior than in Kensington throughout the sampling period (Fig. 2). Significant difference ($P < 0.05$) between cultivars were observed at all time points except at 1 dpi (Table 1). β-1,3-glucanase activity also varied significantly over time in both cultivars ($P < 0.05$).

Phenylalanine Ammonia lyase (PAL) activity showed significant temporal variation after inoculation in both cultivars. In Prior, PAL activity was highest at 1 dpi (Fig. 3A). However, at each specific time interval, no significant difference in PAL activity was observed between Prior & Kensington (Table 2).

Polyphenol oxidase (PPO) activity was significantly higher in Kensington than in Prior at all time points. At 1 and 7 dpi, PPO activity in Kensington was approximately 67% and 85% higher, respectively, compared to Prior (Fig. 3B). Significant differences ($P < 0.05$) in PPO activity were observed between the two cultivars at each time interval (Table 2).

Peroxidase (POD) activity varied significantly over time after inoculation in both cultivars (Fig. 3C). POD activity was significantly higher in Kensington than in Prior at 3, 5, and 7 dpi, whereas no significant difference between cultivars was observed at 0 and 1 dpi (Table 2).

Significant temporal changes in SOD activity were detected in both cultivars following inoculation (Fig. 3D). In Prior, the highest SOD activity was recorded at 3 dpi. However, SOD activity did not differ significantly between Prior and Kensington at any corresponding time point (Table 2).

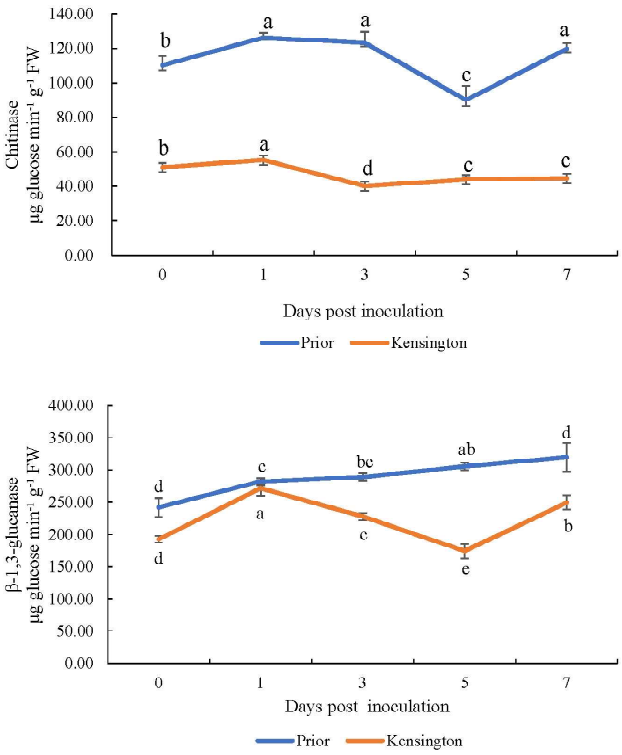

Fig. 2 : Effect of *C. asianum* on the production of Chitinase and β-1,3-glucanase activity in Prior (MR) and Kensington (S) mango genotypes. Values represent as mean ± SD of three independent biological replicates (n = 3). *Different lowercase letters (a–e) indicate significant differences among time points within the same genotype according to DMRT at $p \leq 0.05$

Temporal and genotypic variation in enzyme activity

Two-way ANOVA revealed that enzyme activity was predominantly influenced by time, with significant effects ($p<0.05$) for all enzymes except SOD. β-1,3-Glucanase ($F(4,20)=25.40$, $p=1.27e-07$), Chitinase ($F(4,20)=36.47$, $p=4.22e-09$), PAL ($F(4,20)=23.79$, $p=4.30e-05$), POD ($F(4,20)=69.29$, $p=2.98e-07$), and PPO ($F(4,20)=51.59$, $p=1.22e-06$) all showed statistically significant temporal changes. In contrast, SOD did not show a significant temporal effect ($F(4,20)=2.10$, $p=0.118$).

Significant genotype × time interactions were observed for β-1,3-Glucanase ($F(4,20)=39.65$, $p=1.86e-09$) and chitinase ($F(4,20)=27.80$, $p=5.57e-08$). PAL showed a marginal, non-significant interaction ($F(4,20)=2.79$, $p=0.0854$). Only POD exhibited a significant main effect of genotype ($F(1,20)=4.98$, $p=0.0497$), indicating inherent baseline differences between Prior and Kensington.

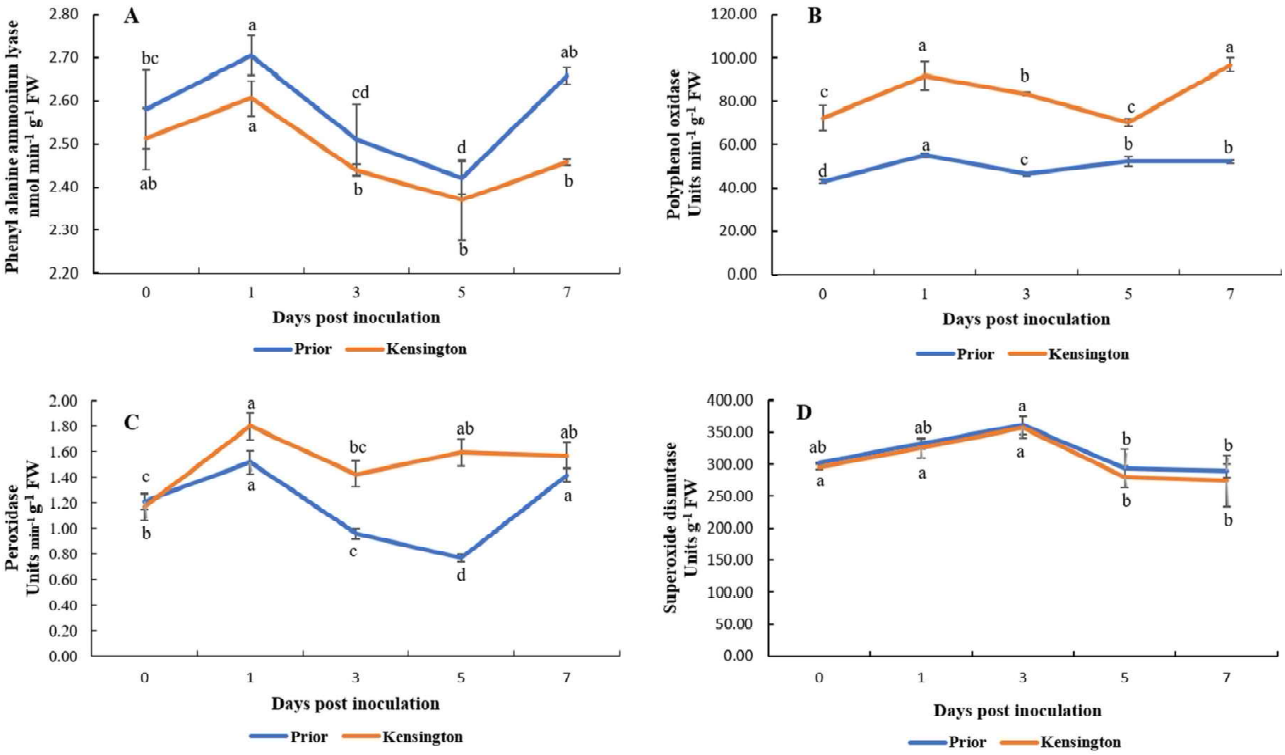

Fig. 3 : Changes in (A) Phenylalanine ammonium lyase, (B) Polyphenol oxidase, (C) Peroxidase and (D) Superoxide dismutase activities in Prior (MR) and Kensington (S) mango fruits inoculated with *C. asianum*. *Data are presented as mean±SD of three independent biological replicates (n=3). Different lowercase letters (a–d) indicate significant differences among time points within the same genotype according to DMRT at $p<0.05$

Correlation analysis of defense-related enzymes

The enzyme correlation matrix (Fig. 4) revealed a strong positive correlation between chitinase and β-1,3-glucanase (r=0.70) and a strong negative correlation between chitinase and PPO (r=-0.85). PPO and POD were positively correlated (r=0.65) in 'Kensington', while SOD showed weak correlations with other enzymes.

Principal component analysis

PCA explained 72.90% of the total variance (PC1=48.95%, PC2=23.95%). Samples from Prior clustered distinctly from Kensington along PC1, indicating genotype as the primary source of variation. PC2 reflected temporal variation, with samples at different dpi occupying distinct positions. Prior showed a more compact clustering pattern, whereas Kensington samples were more dispersed (Fig. 5).

Mango fruit undergoes physiological changes during ripening and senescence, resulting in reduced intrinsic resistance and increased susceptibility to fungal pathogens (Prusky, 1996). This study investigated defense responses in mango genotypes with contrasting resistance to *C. asianum*, focusing on pathogenesis-related proteins, PAL, and oxidative stress enzymes. The synthesis of PR proteins is a fundamental plant defense mechanism following pathogen attack. Chitinase and β-1,3-glucanase activities were significantly higher in Prior than in Kensington. These enzymes hydrolyze the chitin and β-1,3-glucan in fungal cell walls, thereby inhibiting pathogen growth and spread. Similar findings have been reported in mango cv. 'Karutha Colomban' upon infection with *C. gloeosporioides* (Sinniah et al., 2012). In mango, Jimenez-Maldonado et al. (2024) identified distinct expression profiles of GLUC and CHIT class I and IV genes in cv. 'Ataulfo' following infection with *C. siamense* and *C. asianum*. Together, these findings support the role of chitinase and β-1,3-glucanase in limiting fungal invasion and contributing to early defense responses in resistant mango genotypes.

Phenylalanine ammonia lyase activity peaked at 1 dpi in the moderately resistant genotype, followed by a gradual decline. A similar pattern was reported in mango cv. 'Karutha Colomban' (Sinniah et al., 2012), while, Chakraborty et al. (2019) reported that PAL activity peaked at 2 dpi and declined thereafter. Although PAL activity was higher in the moderately

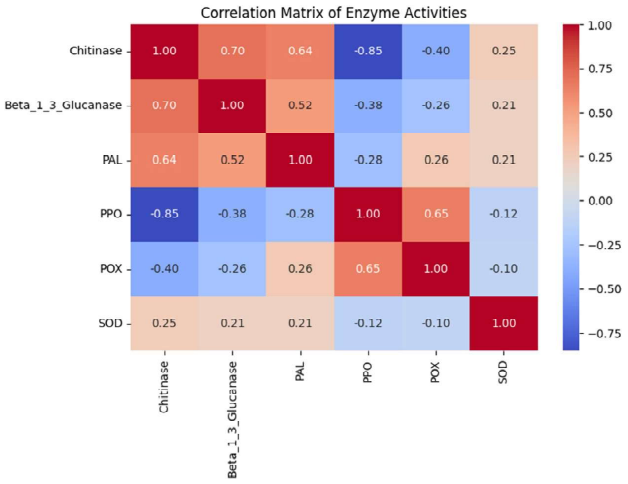

Fig. 4 : Correlation matrix of pathogenesis-related enzymes in response to *C. asianum* infection

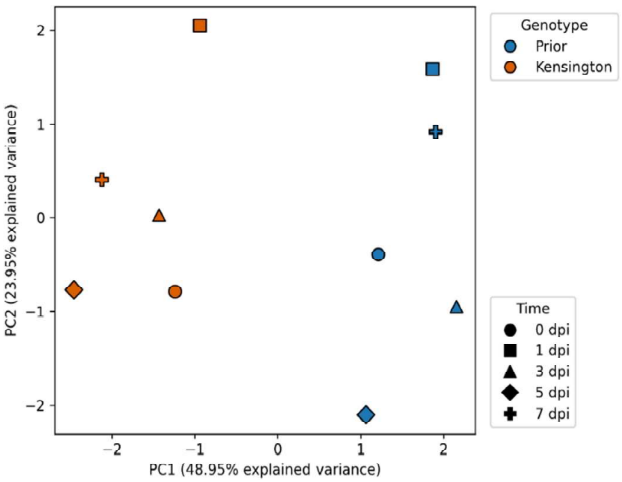

Fig. 5 : Principal component analysis of defense-related enzymatic profiles in response to *C. asianum* in Prior and Kensington genotype

resistant genotype, the difference was not statistically significant, suggesting that PAL may function as part of a general stress response rather than a specific resistance marker (Mahatma et al., 2011). Superoxide dismutase activity increased in both genotypes upon infection but was more pronounced in Prior, indicating a more efficient ROS detoxification system. A similar pattern was observed in walnut, where resistant and susceptible clones both exhibited increased SOD activity, but the magnitude of change differed (Yang et al., 2021). Likewise, increased SOD activity has been observed in both resistant and susceptible melon cultivars following *C. lagenarium* infection, supporting the role of SOD in basal defense (Ge et al., 2013).

Polyphenol oxidase and peroxidase are oxidative enzymes involved in plant defense but can also contribute to enzymatic browning and necrosis when activated at late stages of infection. In the present study, PPO and POD activities were higher in Kensington, suggesting a stress-associated host response. Similarly, Wang et al. (2020) found that enzymatic browning in potato tubers was positively correlated with PPO and POD activity and that PPO gene expression was higher in browning-susceptible cultivars. In pearl millet, POD activity was highest in susceptible genotypes at both pre- and post-infection stages (Mahatma et al., 2011). The negative correlation of PPO with hydrolytic enzymes suggests a lack of coordinated activation among defense-related enzymes. The PPO–POD correlation in Kensington suggests an oxidative response that is not accompanied by proportional activation of hydrolytic enzymes (Apel & Hirt, 2004). Overall, resistance in mango to *Colletotrichum* involves timely activation of hydrolytic enzymes (chitinase, β-1,3-glucanase) and effective antioxidant buffering (SOD). In contrast, susceptible genotypes appear to rely more on oxidative pathways (PPO, POD), which may be insufficient for effective defense.

CONCLUSION

The study highlights the role of various PR proteins and defense related enzymes in mango resistance to *C. asianum*. The elevated activities of chitinase and β-1,3-glucanase in moderately resistant genotypes highlight their significance in enhancing resistance by hydrolyzing fungal cell wall components. In contrast, higher PPO and POD activities in susceptible genotypes indicate a pronounced oxidative response that may be associated with disease progression rather than effective resistance. These findings provide valuable insights into the biochemical interactions between mango and *C. asianum* and offer a foundation for further exploration of host–pathogen relationships and the development of anthracnose resistant cultivars.

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