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Research Article

ENZYMATIC AND BIOCHEMICAL STUDIES OF DEFENCE RESPONSES OF MANGO (*Mangifera indica L.*) HYBRIDS TO LEAFHOPPER INFESTATION

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Abstract- In the present study we evaluated the potential role of antioxidant enzymes and phenols in the defence response of five mango hybrids (*Mangifera indica* L.'Mallika', 'Swarna Jehangir', 'Neeleshan','Neelgoa' and 'Ratna')to leafhopper infestation at two stages of leaf maturity (new flush and old leaves). Changes in activities of antioxidant enzymes namely superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione reductase (GR), polyphenol oxidase (PPO) and ascorbate oxidase (AO) and different biochemical parameters in infested leaves were observed as compared to healthy ones. Strong induction of peroxidase, glutathione reductase and polyphenoloxidase in new flush (88.14%, 89.53% and 78.99%) and old leaves 69.57%, 68.75% and 72.85%) was observed. The accumulation of phenols was preferentially enhanced (new flush-73.99% and old leaves-72.19%). Under infestation total chlorophyll and reducing sugar content were decreased in all the cultivars but to a varying degree. The antioxidant activities of mango leaves was significantly affected by leaf age with higher constitutive and induced levels in new flush as compared to old leaves. Mallika with the highest basal and induced antioxidant enzyme activities is indicated as the most tolerant hybrid whereas Ratna having the lowest is reported as the most sensitive hybrid to leafhopper infestation. Our results implicated that peroxidase, polyphenol oxidase, glutathione reductase and phenols played an important role in integrated defence response of mango to leafhopper infestation and the hybrids with higher levels of tolerance exhibited higher capacity for up regulation of defensive enzymes.

Keywords- Antioxidant enzymes, Mango, Hybrids, Leaf hopper, Phenols, Defense response.

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Introduction

In India, Mango (*Mangifera indica* L.) is the major fruit crop and occupies an area of about 2.52 Mh with 18.4 MT production [1]. Among the pests that occur on mango, leaf hoppers *Amritodus atkinsoni, Idioscopus niveosparsus* and *Ideoscopus clypealis* cause major damage to leaf and inflorescence resulting in drying of the entire inflorescence and ultimately affecting fruit setting thus leading to severe yield and financial loss [2].

Plant resistance offers a promising approach for managing insect pests because it is sustainable and environmentally responsible. In plants, defense reactions to counter or offset the herbivore attack include plant metabolites and macro-molecules (e.g. peptides, proteins, enzymes, lignin, phenolic metabolites, cuticular waxes and tannins etc.) [3].The transient production of reactive oxygen species (ROS) in an oxidative burst is frequently an early plant response to pathogen attack [4]. ROS have been suggested to be involved in defense responses in several ways by acting as toxic agents, participating as secondary messengers via transduction pathways, which have H_2O_2 as a secondary messenger leading to the activation of plant defense related genes and reinforcing plant cell walls through cross linking reactions of lignins and proteins [5]. Over a certain level however ROS may have deleterious effects to plant cells through oxidative damage to lipids, proteins and nucleic acids. To keep the levels of ROS under control plants have non-enzymatic and enzymatic antioxidants that operate together to protect cells from oxidative damage [6]. The antioxidant enzymes involve superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione reductase (GR), ascorbate oxidase (AO), polyphenol oxidase (PPO) among others. These antioxidant enzymes and secondary metabolites because of their potential roles in synthesis of defense compounds and oxidative stress tolerance have been implicated in plant resistance to insect herbivory [7].

The capability of host systems to maintain redox balance under insect attack may correlate with the resistance of plants to infestation. The rapid and efficient functioning of complex cellular antioxidative networks might be an important factor in the ability to alleviate the leaf hopper stimulated oxidative burst and thus may be the basis of greater ability of host to survive infestation. With these objectives the present study was undertaken to biochemically characterize the defense response of 5 mango hybrids to leaf hopper infestation by scrutinizing the antioxidative enzyme activities and phenolic content, reducing sugars and chlorophyll.

Materials and Methods

Plant Material

Five hybrids of mango (*Mangifera indica*) namely 'Mallika' (Neelum x Dashehari) developed at Indian Agricultural Research Institute, New Delhi, India; 'Swarna Jehangir' (Chinnaswarnarekha x Jehangir); 'Neelgoa' (Neelum x Yerramulgoa); 'Neeleshan' (Neelum x Baneshan); 'Ratna' (Neelum x Alphonso) released at Forest Research Station, Kodur, Andhra Pradesh, India [8] were selected for the study to characterize biochemical mechanisms conferring the resistance to leaf hopper.

Leafhopper infested and healthy leaves were collected from the selected mango hybrids from Silver Jubilee Orchard, University of Agricultural Sciences, Dharwad,

at two stages of leaf maturity i.e., new flush (25-35 days) and old leaves (120-130 days). Leaf tissues were processed immediately for enzyme extraction and assay. All the processing steps were carried out at 0˚C-4˚C.

Extraction of enzyme and determination of protein content

In order to measure the antioxidant enzyme activities (SOD, CAT, POX, GR, AO and PPO), fresh leaf samples (1g) were taken per treatment and ground into fine powder using liquid nitrogen and homogenized in a pre–chilled mortar with 4.0 mL of ice cold buffer of specific composition, molarity and pH for each antioxidant enzyme [9]. CAT and SOD were extracted in 0.05M sodium phosphate buffer of pH 7.0 and pH 7.8, respectively. AO, PPO and POX extracts were prepared in 0.1M potassium phosphate buffers of pH 5.6, 6.8 and 7.0, respectively. Grinding buffer for GR contained 0.1M Tris –HCl pH 7.8 and 2mM dithiothreitol (DTT). 1mM ethylenediaminetetraacetic acid (EDTA) and 1.5% w/v insoluble polyvinyl polypyrrolidone were used in all extraction buffers. The homogenate was centrifuged at 14,000 rpm for 20 min at 4 ˚C, and the supernatant was used immediately as an enzyme source for the assay. An aliquot of supernatant was stored at -20˚C for protein analysis. The protein content in the extract was determined by Bradford method using bovine serum albumin as standard [10].

Antioxidant enzyme activities

Superoxide dismutase Assay-The activity of SOD, (EC 1.15.1.1) was assayed spectrophotometrically at 560 nm [11]. 3.0 mL of reaction mixture contained 20 µL of enzyme extract, 10mM L-methionine, 33 µM p-nitrobluetetrazolium chloride (NBT), 0.66 µM EDTA and 3.3 µM riboflavin in a 50mM potassium phosphate buffer, pH 7.8. The reaction was initiated by adding riboflavin and took place in a chamber illuminated by a 15W fluorescent lamp at 25˚C for 15 minutes. The blue formazan produced by NBT photo-reduction was measured by the increase in absorbance at 560 nm. One unit of SOD is defined as the amount of enzyme required to inhibit 50% of the NBT photo-reduction per minute and expressed as IU per mg protein.

Catalase Assay: CAT (EC 1.11.1.6) activity was determined spectrophotometrically [12]. The reaction was initiated by adding 20 µL of enzyme extract to 2.98 mL of 16.65 mM hydrogen peroxide in 50mM phosphate buffer, pH 7.0. The decrease in absorbance at 240 nm was measured for 5 minutes. One unit of CAT is defined as the one µmole of H_2O_2 decomposed per minute at pH 7.0 at 25˚C and was expressed as µmole $min^{-1}mg^{-1}$ protein.

Peroxidase Assay: POX (EC 1.11.1.7) activity was determined spectrophotometrically [13]. The reaction was initiated by adding 20 µL of the enzyme preparation to reaction mixture containing 2.88 mL of 100mM potassium phosphate buffer, pH 7.0, 50 µL of 20 mM guaiacol, 50 µL of 0.042% H_2O_2 and increase in absorbance was monitored at 436 nm for 5 minutes. One POX unit is defined as the amount of enzyme which catalyses the formation of one micromole of oxidized guaiacol per minute at 25°C and expressed as µmole $min^{-1}mg^{-1}$ protein.

Glutathione reductase Assay. GR (EC 1.8.1.7) activity was spectrophotometrically determined [14]. The decrease in absorbance at 340 nm on addition of 100 µL of enzyme to reaction mixture containing 1.5 mL of 100 mM potassium phosphate buffer with 3.4 mM EDTA, pH 7.6, 100 µL of 30 mM oxidized glutathione, 350 µL of 0.8 mM ß-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and 950 µL of water was recorded for 5 minutes. One GR unit is defined as the amount of enzyme that oxidises 1.0 µmole of NADPH per minute at pH 7.6 at 25°C and expressed as µmole $min^{-1}mg^{-1}$ protein.

Ascorbate oxidase Assay: AO (EC 1.10.3.3.) activity was assayed spectrophotometrically [15]. The reaction was initiated by adding 20 µL of crude enzyme extract to the assay mixture containing 2.98 mL of 0.1M Phosphate / 0.5mM EDTA buffer, pH5.6, 100 µL of 5mM ascorbic acid and the decrease in absorbance at 265 nm was recorded over 8 minutes. One unit of AO is defined as the amount of enzyme catalysing the oxidation of 1 micromole of ascorbate per minute at 25°C and expressed as µmole min^{-1} mg^{-1} protein.

Polyphenol oxidase Assay: PPO (EC 1.10.3.1) activity was spectrophotometrically determined [16]. The reaction mixture contained 0.5 mL of 100mM catechol solution in 2.48 mL of 100mM phosphate buffer, pH 6.8. 20 µL of enzyme extract was used to initiate the reaction which was measured by increase in absorbance at 410 nm with 15 sec interval up to 5 minutes. One PPO unit is defined as the amount of enzyme that increased the absorbance by 0.001 per minute under the conditions of the assay and expressed as µmole min^{-1} mg^{-1} protein.

Determination of total phenols, reducing sugars and chlorophyll

Total Phenols- One gram of dried leaf tissue was extracted with 10 mL of hot 80% ethanol [17]. The colorimetric method [18] used for the determination of total phenols using the Folin–Ciocalteau reagent. The phenol content was expressed as mg per gram dry weight.

Reducing sugars-For quantitative estimation of reducing sugars one gram dry weight of leaf material was plunged in 10 mL of hot 80% ethanol for 5 min and then crushed in pestle and mortar. The slurry thus obtained was filtered and the residue was re-extracted two or three times and the filtrate were made up to 10 mL with 80% ethanol. Reducing sugars were measured by Nelson's modification of Somogi's method [19]. The reducing sugar was expressed as mg per gram dry weight.

Chlorophyll-Chlorophyll content present in leaf samples was determined by Dimethyl sulphoxide (DMSO) method [20]. The values obtained were expressed as mg per gram fresh weight.

Statistical Analysis

The data of the experiment was analyzed statistically following the procedure described by Gomez and Gomez [21].The experimental design followed a three factorial complete randomized system containing three replicates. The results were expressed as mean and standard error of mean of three replicates of the enzyme assay for each sample. The data was analyzed by analysis of variance (ANOVA) and the means were compared using (C.D.) at the 1% probability.

Results

Changes in activities of antioxidant enzymes in mango leaves due to leaf hopper infestation.

Superoxide dismutase activity. SOD activity of mango hybrids differed significantly ($p \leq 0.01$) in healthy and infested leaves **[Fig-1].** Biotic stress due to leaf hopper infestation induced SOD activity in new flush of Mallika, Swarna Jehangir, Neelgoa and Neeleshan hybrids however in Ratna loss of activity was observed (943.23, 859.54, 693.22, 602.89 and 338.95 IUmg-1 protein, respectively). The basal SOD activity in the Mallika and Swarna Jehangir was significantly higher (843.83 $IUmg^{-1}$ protein and 757.89 $IUmg^{-1}$ protein respectively) compared to all other hybrids whereas Ratna recorded the lowest basal activity (451.76 $IUmg^{-1}$ protein). A similar trend was observed in old healthy and infested leaves but the degree of response in both constitutive and induced activity was significantly lower compared to new flush.

Catalase activity: CAT activity was altered significantly ($p \leq 0.01$) in all the cultivars by the stress imposed by leaf hopper infestation **[Fig-2].** The CAT activity increased in infested leaves of all the hybrids except in Ratna. In new flush infested leaves, Mallika and SwarnaJehangir had significantly greater CAT activity (58.83 and 48.40 µmol $min^{-1}mg^{-1}$protein, respectively) followed by Neelgoa and Neeleshan (46.44 and 44.68 µmol $min^{-1}mg^{-1}$protein) though a greater increase was observed for Neelgoa (39.66%) than Mallika (26.50%) in old leaves. Following infestation Ratna showed drastic reduction in CAT activity as compared to new flush healthy leaves (decrease of 27.06%).The biochemical response of old healthy and infested leaves of all the hybrids was significantly lower than new flush leaves

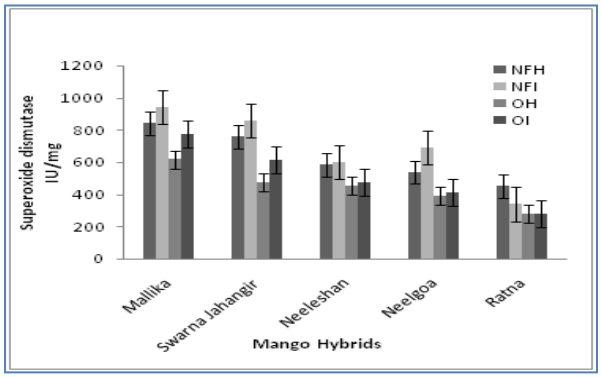

Fig-1 Specific activity of Superoxide dismutase (SOD) (IU mg^{-1} protein) in the leaves of five mango Hybrids Mallika, Swarna Jehangir, Neeleshan, Neelgoa and Ratna under leafhopper infestation. The values are the means of three replicates ±SEM. NFH – New flush healthy. NFI – New flush infested. OH – Old healthy. OI – Old infested.

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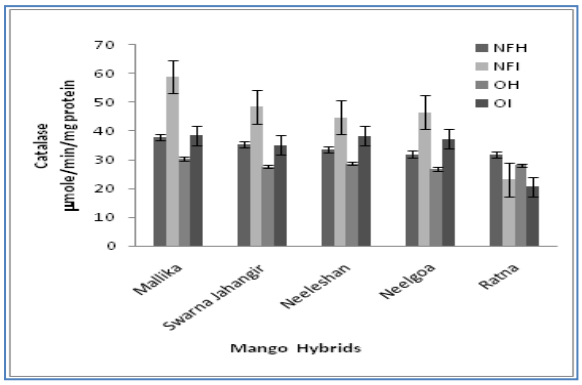

Fig-2 Specific activity of Catalase (CAT) (µmol min^{-1}mg^{-1} protein) in the leaves of five mango Hybrids Mallika, Swarna Jehangir, Neeleshan, Neelgoa and Ratna under leafhopper infestation. The values are the means of three replicates ± SEM. NFH – New flush healthy. NFI – New flush infested. OH– Old healthy. OI–Old infested

Peroxidase activity: Infested leaves of all the hybrids showed a significant (p≤ 0.01) induction of POX activity compared with the healthy leaves **[Fig-3].** Analysis of POX activity in hybrids indicated strong induction under infestation stress for Mallika, Neeleshan, Swarna Jahangir, Neelgoa and Ratna (1.11, 0.4, 0.39, 0.38 and 0.23 µmol min^{-1}mg^{-1} protein respectively) which differed significantly from healthy leaves (0.59, 0.33, 0.28, 0.18 µmol min^{-1}mg^{-1} protein respectively). In new flush infested leaves the highest POX activity was observed in Mallika and recorded an increase of 88.14%. Other hybrids showed higher POX activity in infested leaves but the height of response was lower compared to Mallika. Contrary to the other anti-oxidative enzymes, peroxidase activity increased in Ratna both in new flush and old leaves. Similar response was observed in old leaves.

Glutathione reductase activity: GR activity of mango hybrids differed significantly (p≤ 0.01) in healthy and infested leaves of both new flush and old leaves **[Fig-4].** In new flush infested leaves, Mallika and Swarna Jehangir had significantly greater GR activity (13.40 and 10.44 µmol min^{-1}mg^{-1} protein respectively) followed by Neeleshan and Neelgoa (9.95 and 8.3 µmol min^{-1}mg^{-1} protein respectively) in comparison with healthy leaves (7.07, 6.34, 5.79 and 5.6 µmol min^{-1}mg^{-1} protein respectively). The greatest induced activity was observed in Mallika in new flush (increase of 89.53%) whereas in old leaves Neelgoa gave the maximum response (increase of 68.75%).Ratna showed decrease in GR activity in both infested new flush and old leaves (7.59% and 25.00 % decrease respectively). Constitutive levels of GR activity were lowest in Ratna (3.82 µmol min^{-1}mg^{-1}protein) and highest in Mallika (7.07 µmol min^{-1}mg^{-1} protein) in new flush. Similar trend was observed in old leaves.

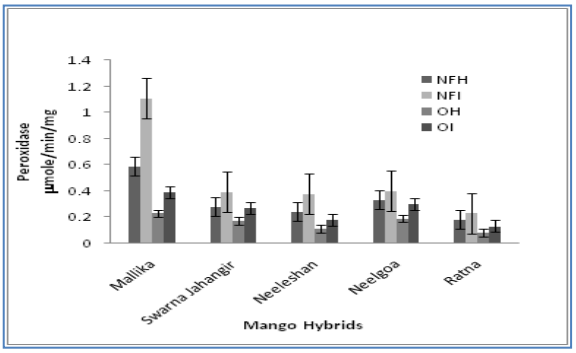

Fig-3 Specific activity of Peroxidase (POX) (µmol min^{-1}mg^{-1} protein) in the leaves of five mango Hybrids Mallika, Swarna Jehangir, Neeleshan, Neelgoa and Ratna under leafhopper infestation . The values are the means of three replicates ± SEM. NFH – New flush healthy. NFI – New flush infested. OH – Old healthy. OI – Old infested.

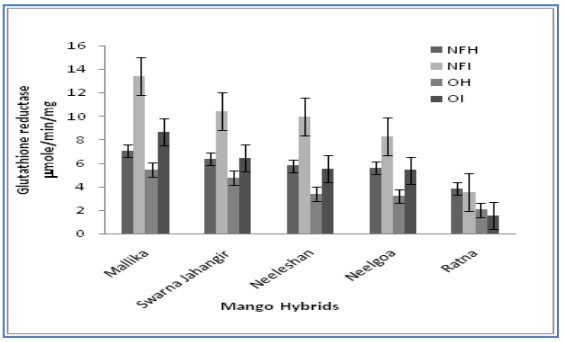

Fig-4 Specific activity of Glutathione Reductase (GR) (µmol min^{-1}mg^{-1} protein)in the leaves of five mango Hybrids, Mallika, Swarna Jehangir, Neeleshan, Neelgoa and Ratna under leafhopper infestation. The values are the means of three replicates ± SEM. NFH – New flush healthy. NFI – New flush infested. OH – Old healthy. OI - Old infested.

Ascorbate oxidase: The AO activities in new flush and the old leaves were significantly (p≤ 0.01) greater in the infested leaves in comparison with the healthy leaves **[Fig-5].** Analysis of AO activity in hybrids indicated induction under infestation stress for Mallika, SwarnaJahangir, Neeleshan, Neelgoa (3.57, 2.71, 1.81 and 1.50 µmol min^{-1}mg^{-1}protein respectively) which differed significantly from healthy leaves (2.17, 1.89, 1.17 and 0.97 µmol min^{-1}mg^{-1} protein respectively). Ratna recorded decrease in AO activity in infested leaves (0.72 µmol min^{-1}mg^{-1}protein) compared to healthy leaves (1.04 µmol min^{-1}mg^{-1} protein). Among the hybrids Mallika and Neelgoa showed maximum increase in AO activity (64.58 % and 54.64% increase respectively). The basal activity of Mallika was 2.3 folds higher than Ratna and all the genotypes exhibited higher basal and induced activity in new flush compared to old leaves.

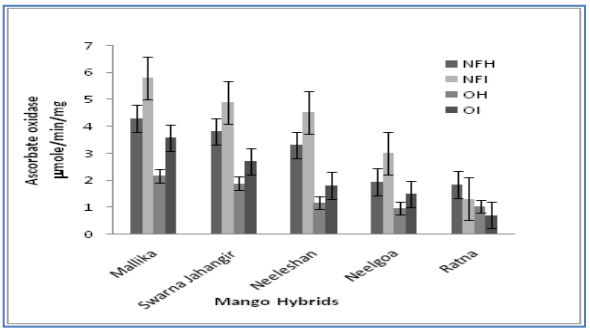

Fig-5 Specific activity of Ascorbate oxidase (AO) (µmol min^{-1}mg^{-1} protein) in the leaves of five mango Hybrids, Mallika, Swarna Jehangir, Neeleshan, Neelgoa and Ratna under leafhopper infestation. The values are the means of three replicates ± SEM. NFH – New flush healthy. NFI – New flush infested. OH – Old healthy. OI - Old infested

Polyphenol oxidase activity: Leaf hopper infestation had profound effect on PPO activity in all the hybrids **[Fig-6].** With respect to hybrids, Mallika recorded significantly higher PPO activity in infested leaves (4.10 µmol min^{-1} mg^{-1} protein) followed by Neelgoa (2.37 µmol min^{-1} mg^{-1} protein) , Swarna Jehangir (1.85 µmol min^{-1} mg^{-1} protein) and Neeleshan (1.69 µmol min^{-1} mg^{-1} protein) when compared to healthy leaves in new flush. However in Ratna the opposite effect, decrease of 39% and 58.49 % in PPO activity was observed in infested new flush and old leaves respectively. Mallika recorded maximum induction (78.99%) in new flush infested leaves. The basal activities of PPO in new flush ofall the hybrids were nearly 2 folds higher than in old leaves. Induced PPO activities of Mallika, Neelgoa and SwarnaJehangir hybrids were a little higher in old leaves compared to new flush.

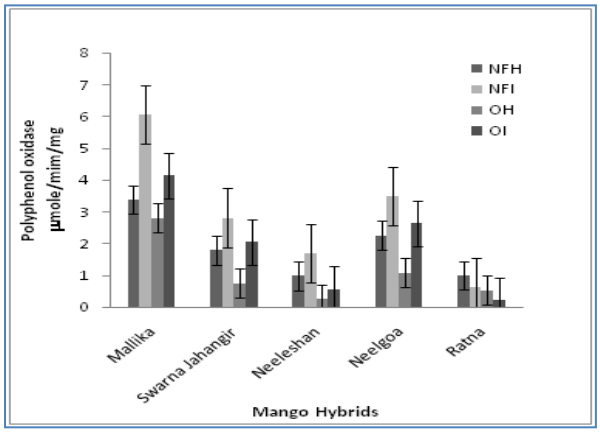

Fig-6 Specific activity of Polyphenol oxidase (PPO)(µmol $min^{-1}mg^{-1}$ protein) in the leaves of five mango Hybrids Mallika, Swarna Jehangir, Neeleshan, Neelgoa and Ratna under leafhopper infestation. The values are the means of three replicates ± SEM. NFH – New flush healthy. NFI –New flush infested. OH –Old healthy. OI -Old infested

Changes in phenols, reducing sugars and total chlorophyll content of mango leaves due to leaf hopper infestation.

Total Phenols: We observed that phenol content in the leaves of all mango hybrids, differed significantly ($p \leq 0.01$) under healthy and infested condition caused by mango leafhopper **[Fig-7].** Among hybrids, Mallika, recorded significantly higher phenol content under infestation (72.68 mg/g dry weight) than in healthy leaves (60.68 mg/g dry weight) however Neeleshan, Swarna Jehangir and Neelgoa exhibited maximum induction (63.33, 62.74, and 64.65 mg/g dry weight respectively)compared to control (36.15, 37.08 and 37.30 mg/g dry weight respectively). Total phenols were reduced under infestation in Ratna (16.67 % and 10.87% decrease in new flush and old leaves respectively). Similar trend was observed in old leaves.

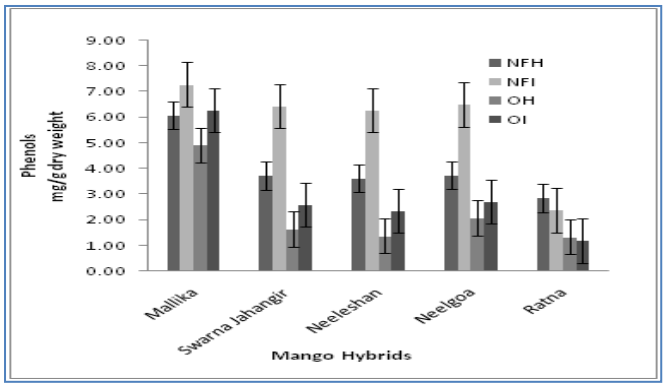

Fig-7 Phenols (mg/g dry weight) in the leaves of five mango Hybrids, Mallika, Swarna Jehangir, Neeleshan, Neelgoa and Ratna under leafhopper infestation. The values are the means of three replicates ± SEM. NFH – New flush healthy. NFI – New flush infested. OH – Old healthy. OI - Old infested.

Reducing sugar content: In the present study total reducing sugar content was decreased significantly ($p \leq 0.01$) in infested leaves as compared to healthy leaves in all the hybrids **[Fig-8].** Leaf hopper infestation caused higher decrease of reducing sugars in old leaves (35%%-64%) than in new flush (1%- 41%). Swarna Jehangir followed by Neelgoa, Neeleshan, Ratnaand Mallika recorded significantly higher reducing sugars in the control (21.03, 21.33, 23.10, 21.21 and 19.75 mg/g dry weight respectively) than in the infested condition (10.78, 10.43, 12.77, 9.79 and 8.20 mg/g dry weight respectively).Similar trend was observed in old leaves.

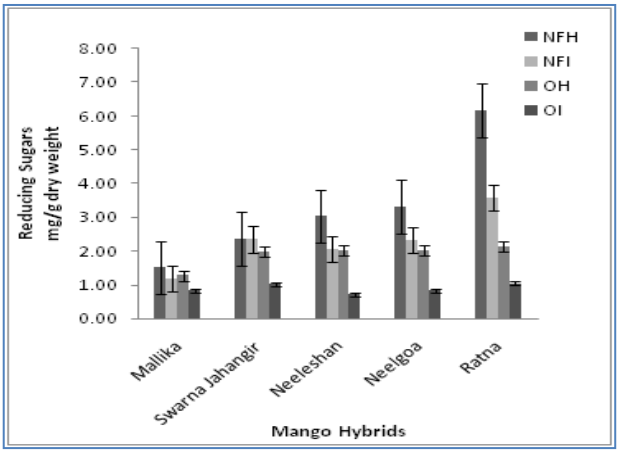

Fig-8 Reducing sugars (mg/g dry weight) in the leaves of five mango Hybrids, Mallika, Swarna Jehangir, Neeleshan, Neelgoa and Ratna under leafhopper infestation. T he values arethe means of three replicates ± SEM. NFH–New flush healthy. NFI – New flush infested. OH – Old healthy. OI – Old infested.

Total chlorophyll: Leaf chlorophyll content was significantly affected by leaf hopper infestation in all the hybrids **[Fig-9].** Infested leaves had lower chlorophyll content than the healthy leaves (decreased by 13%-50%). The reduction in chlorophyll content was highest in Ratna (up to 50% decrease) while all the other hybrids showed 13% to 36% decrease in chlorophyll content in both the new flush and old leaves.

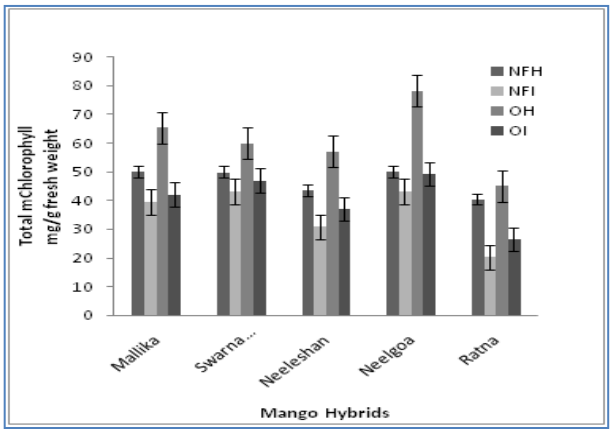

Fig-9 Total Chlorophyll (mg/g fresh weight) in the leaves of five mango Hybrids, Mallika, Swarna Jehangir, Neeleshan, Neelgoa and Ratna under leafhopper infestation. The values are the means of three replicates ± SEM. NFH – New flush healthy. NFI – New flush infested. OH – Old healthy. OI – Old infested.

Discussion

Changes in activities of antioxidant enzymes in mango leaves due to leaf hopper infestation.

Superoxide dismutase activity: Superoxide dismutases, a group of metalloenzymes, are considered as the first defence against ROS being responsible for the dismutation of $O_2^-$to H_2O_2 and O_2. The results demonstrated

the variability in the extent of the anti-oxidative capacity in terms of SOD activity. The differences in the expression profiles of several *sod* genes have been demonstrated resulting in marked variation in SOD activities in different maize varieties in response to aphid infestation [22]. The present findings suggest that leafhopper infestation might have relatively up-regulated the expression and activation of *sod* genes in Mallika, Swarna Jehangir and Neelgoa hybrids than in Neeleshan and Ratna. The decreased SOD activity in Ratna indicates its inactivation due to the accumulated ROS induced by infestation and low basal SOD activity. The differential behaviour observed in the SOD activity of the hybrids could also be related to the different subcellular distribution of SODs along with the different isoenzyme sensitivity. Mallika and Swarna Jehangir are suggested to be more efficient in their defense against leafhopper than the other hybrids in lieu of higher constitutive as well as greater SOD induced activity. The decline in SOD activity was observed in leaf tissue on maturity. The lower SOD activity in old leaves may indicate the lower synthesis of SOD at this stage compared to the new flush and hence present greater vulnerability to ROS.

Table-1 *Effect of leafhopper infestation on superoxide dismutase activity in mango leaf at different stages of leaf growth*

Genotypes	New Flush			Old Leaves		
	Healthy (H)	Infested (I)	%decrease or increase in I over H	Healthy	Infested	% decrease or increase in I over H
Mallika	843.83	943.23	11.78	617.54	773.33	25.23
Swarna Jehangir	757.89	859.54	13.41	474.25	612.90	29.24
Neeleshan	583.39	602.89	3.23	453.02	475.61	4.99
Neelgoa	537.97	693.22	28.86	393.85	413.88	5.09
Ratna	451.76	338.95	-24.97	278.29	278.29	0.00

Table-2 *Effect of leafhopper infestation on Catalase activity in mango leaf at different stages of leaf growth*

Genotypes	New Flush			Old Leaves		
	Healthy (H)	Infested (I)	%decrease or increase in I over H	Healthy (H)	Infested (I)	% decrease or increase in I over H
Mallika	37.82	58.83	55.55	30.40	38.46	26.50
Swarna Jahangir	35.33	48.40	37.00	27.61	35.04	26.92
Neeleshan	33.58	44.68	33.05	28.70	38.35	33.61
Neelgoa	31.95	46.44	45.36	26.68	37.26	39.66
Ratna	31.67	23.10	-27.06	28.07	20.68	-26.33

Table-3 *Effect of leafhopper infestation on Peroxidase activity in mango leaf at different stages of leaf growth*

Genotypes	New Flush			Old Leaves		
	Healthy (H)	Infested (I)	%decrease or increase in I over H	Healthy	Infested	% decrease or increase in I over H
Mallika	0.59	1.11	88.14	0.23	0.39	69.57
Swarna Jahangir	0.28	0.39	39.29	0.17	0.27	58.82
Neeleshan	0.24	0.38	58.33	0.11	0.18	63.64
Neelgoa	0.33	0.4	21.21	0.19	0.30	57.89
Ratna	0.18	0.23	27.78	0.08	0.13	62.50

Table-4 *Effect of leafhopper infestation on Glutathione Reductase activity in mango leaf at different stages of leaf growth*

Genotypes	New Flush			Old Leaves		
	Healthy (H)	Infested (I)	%decrease or increase in I over H	Healthy	Infested	% decrease or increase in I over H
Mallika	7.07	13.40	89.53	5.45	8.63	58.35
Swarna Jehangir	6.34	10.44	64.67	4.75	6.43	35.37
Neeleshan	5.79	9.95	71.85	3.36	5.54	64.88
Neelgoa	5.60	8.30	48.21	3.20	5.40	68.75
Ratna	3.82	3.53	-7.59	2.04	1.53	-25.00

Catalase activity: Hydrogen peroxide scavenging in plants is essential for cellular protection and cellular signaling [6]. Catalases are the major H_2O_2 scavengers that remove the bulk of cellular H_2O_2 and variation in their levels in plants allow regulation of H_2O_2. The SOD and CAT activities in the infested leaves were higher than those in healthy leaves, suggesting that the protective enzyme reactions in mango leaves were a systematical response to the stress. The increase in CAT activity observed in all hybrids except in Ratnamay be related to increased level of infestation tolerance. Reduced catalase activity in Ratna indicated increased sensitivity to leafhopper induced oxidative stress. The contrasting differences between the Mallika, other hybrids and Ratna may be the result of genetic differences in their metabolic pathways to scavenge oxidative radicals. The biochemical response of old healthy and old infested leaves of all the hybrids was significantly lower than new flush leaves which might be related to the presence of multiple catalase isoenzymes implicating multiple functions for catalases in a variety of plant tissues at various developmental stages and under constantly changing environments [23].

Peroxidase activity: Peroxidase is an important defensive enzyme in plants against a number of biotic and abiotic stresses [24]. In the present study peroxidase activity increased in all the hybrids under leaf hopper stress condition with Mallika recording the highest induction followed by Neelgoa, Swarna Jehangir and Neeleshan. The marked elevation of peroxidase activity in Mallika might be associated with leafhopper resistance. Increased phenol concentration in Mallika might have also increased the peroxidase reaction by acting as other substrate along with H_2O_2, leading to enhanced oxidation of phenolics into reactive quinones whose final products are considered to be anti-nutritive [25]. In Ratna increased peroxidase enzyme activity was observed in contrast to SOD, CAT enzymes which showed decreased activity under infestation. Hence, in Ratna, POX is presumed to play a major role in defense against leafhopper infestation.An increase in POX activity may help to detoxify the peroxides, thus reducing plant

tissue damage under infestation. Higher activity of peroxidase has been linked with reduced insect growth and development in many plants [26, 24]. Peroxidases are the key enzymes in plant cell wall-building processes, lignification, and suberization leading to anti herbivory [27]and thus play a critical role in the plant's defense system. The results of the current study are in conformity with previous studies where insect infestation has been reported to strongly induce peroxidases and suggest that increase in POX activity is a general defensive mechanism in mango hybrids against leaf hopper infestation. The constitutive activity and induced activities of POX were lower in old leaves than in new flush, which indicate there may be an age related differential response to stress due to the selective activation of individual members of gene family in various vegetative and reproductive organs during growth and differentiation.

Table-5 *Effect of leafhopper infestation on Ascorbate oxidase activity in mango leaf at different stages of leaf growth*

Genotypes	New Flush			Old Leaves		
	Healthy (H)	Infested (I)	%decrease or increase in I over H	Healthy (H)	Infested (I)	% decrease or increase in I over H
Mallika	4.30	5.80	34.88	2.17	3.57	64.58
Swarna Jahangir	3.81	4.89	28.35	1.89	2.71	43.39
Neeleshan	3.31	4.51	36.25	1.17	1.81	54.70
Neelgoa	1.94	3.00	54.64	0.97	1.50	54.64
Ratna	1.85	1.32	-28.65	1.04	0.72	-30.77

Table-6 *Effect of leafhopper infestation on Polyphenol oxidase activity in mango leaf at different stages of leaf growth*

Genotypes	New Flush			Old Leaves		
	Healthy (H)	Infested (I)	%decrease or increase in I over H	Healthy	Infested	% decrease or increase in I over H
Mallika	3.38	6.05	78.99	2.81	4.14	47.33
Swarna Jahangir	1.79	2.81	57.07	0.75	2.05	172.85
Neeleshan	0.98	1.69	72.84	0.26	0.57	119.23
Neelgoa	2.26	3.49	54.38	1.08	2.63	143.63
Ratna	1.00	0.61	-39.00	0.53	0.22	-58.49

Table-7 *Effect of leafhopper infestation on Phenols in mango leaf at different stages of leaf growth*

Genotypes	New Flush			Old Leaves		
	Healthy (H)	Infested (I)	%decrease or increase in I over H	Healthy	Infested	% decrease or increase in I over H
Mallika	60.68	72.68	19.79	49.13	62.74	27.71
Swarna Jehangir	37.08	64.33	73.47	16.36	25.82	57.87
Neeleshan	36.15	62.74	73.55	13.63	23.47	72.17
Neelgoa	37.30	64.90	73.99	20.68	27.06	30.83
Ratna	28.35	23.63	-16.67	13.23	11.79	-10.87

Glutathione reductase activity: The regulation of cytosolic redox environment is vital for cell endurance which is largely maintained by glutathione reductase GR- a flavoprotein oxidoreductase, NADPH dependent cellular enzymatic antioxidant and an important component of H_2O_2 scavenging Ascorbate-Glutathione cycle [28]. The major involvement of GR in conferring stress tolerance is the recycling of GSH and maintaining the GSSH/GSG ratio in plant cell. According to our data, GR activity increased under infestation in both new flush and old leaves in the all the hybrids, except in Ratna. The highest levels of constitutive and inducible GR activities exhibited in Mallika and Neelgoa may be related to upregulation of different tissue specific isoforms of GR. Various subcellular isoforms of GR including cytoplasmic, mitochondrial and chloroplastic have been reported in several plant species and the transcript levels of these isofoms fluctuate with environmental factors [29]. Plants over expressing different isoforms of GR showed improved oxidative tolerance in different plants [30,31]. The decrease in GR activity in Ratna leads to a decrease in ascorbate and glutathione pools which may alter the redox balance towards the oxidative state and may be in part related to leafhopper induced promotion of the disease in the susceptible hybrids. During leaf maturation, changes in the oxidative metabolism of plant tissues occur [32], which may be in part related to the difference in GR activity observed in new flush and old leaves.

Table-8 *Effect of leafhopper infestation on Reducing sugars in mango leaf at different stages of leaf growth*

Genotypes	New Flush			Old Leaves		
	Healthy (H)	Infested (I)	%decrease or increase in I over H	Healthy (H)	Infested (I)	% decrease or increase in I over H
Mallika	15.14	11.93	-21.20	12.77	8.20	-35.79
Swarna Jahangir	23.73	23.48	-1.05	19.75	10.11	-48.81
Neeleshan	30.36	20.49	-32.51	20.29	7.24	-64.32
Neelgoa	33.17	23.26	-29.88	20.29	8.20	-59.59
Ratna	61.67	35.85	-41.86	21.23	10.43	-50.86

Table-9 *Effect of leafhopper infestation on Total Chlorophyll in mango leaf at different stages of leaf growth*

Genotypes	New Flush			Old Leaves		
	Healthy (H)	Infested (I)	%decrease or increase in I over H	Healthy	Infested	% decrease or increase in I over H
Mallika	50.01	39.44	-21.14	65.41	42.12	-35.61
Swarna Jahangir	49.88	43.03	-13.72	60.03	47.01	-21.69
Neeleshan	43.61	30.82	-29.33	57.09	37.02	-35.16
Neelgoa	50.12	43.11	-13.99	78.36	49.39	-36.97
Ratna	40.51	20.21	-50.11	45.03	26.40	-41.37

Ascorbate oxidase activity: Ascorbate is the major water soluble antioxidant in plants and animals and is an essential nutrient for most of the insect herbivores. Therefore AO has been proposed to function as a plant defense that decreases the availability of ascorbate to insects [33]. The enzyme oxidizes L-ascorbic acid using molecular oxygen in a two-step reaction to form dehydro-L-ascorbic acid. By selectively decreasing the both oxygen and ascorbic acid content in the apoplast, AO works on both side of the oxidative stress[34]. The regulation of the apoplastic redox state is key to induce plant response to both biotic and abiotic stress [35]. In the current study analysis of AO activity in hybrids indicated strong induction under infestation stress for Mallika, Swarna Jehangir, Neeleshan, Neelgoa. Similar reports of upregulation of AO by herbivory or wounding was reported in various plants [36,37]. Ratna showed a negative response with decreased activity under infestation in both new flush and old leaves. The differential responses of AO enzyme may be one of the possible mechanisms of the differences in infestation sensitivities of the mango Hybrids. The accumulation and export of products changes throughout leaf development [32]. Therefore, the bioactive compounds and the antioxidant activity of leaves in new flush and old leaves differ significantly similar to the findings in the present study where in marked variation in AO activity was observed.

Polyphenol oxidase activity: The polyphenoloxidase plays an important antinutritive role in plant defence against plant pathogen/pest interactions [38]. PPOs catalyze the oxygen dependent oxidation of phenols to quinones, reactive species that can covalently modify and cross link a variety of cellular nucleophiles including side chains of aminoacids leading to cross linking of proteins, and thereby reducing their availability to the insect pests [39]. Our studies showed induction of polyphenol oxidase activity in infested leaves of all the hybrids except in Ratna. Strong induction of PPO and other defenses by methyl jasmonate, system in and oligogalacturonic acid, major plant defense signaling compounds and down regulation of PPO expression resulting in hyper susceptibility to pathogen, suggest a critical role for PPO mediated phenol oxidation in plant defense [40]. Mallika ,Swarna Jahangir, Neelgoa and Neeleshan with higher PPO activity in leafhopper infested leaves supported the role of PPO in defense against leaf eating insects. Ratna showed decreased PPO activity indicating greater susceptibility to leafhopper feeding however, other possible mechanisms like activation of other related enzyme systems (arginase or threonine deaminase) may also be part of the defense system to leaf hopper. The basal and induced activity of PPO in the cultivars were higher in new flush when compared to old leaves suggesting higher phenolic content in new flush may correlate with higher PPO activity and may also reflect the utilization of distinct signal transduction systems for activation of specific PPO isoforms [41].

Changes in phenols, reducing sugars and total chlorophyll content of mango leaves due to leaf hopper infestation.

Phenols: Phenolic compounds are an important component of the oxidative defenses of plants against herbivore s[42]. It has been proposed that phytophenolics, especially flavonols and phenylopropanoids of vacuoles and the apoplast, can detoxify H_2O_2 as electron donors for phenol peroxidases (guaiacol peroxidases) localized in these compartments, which results in the formation of respective phenoxyl radicals. In vitro studies have shown that flavonoids can directly scavenge molecular species of active oxygen. In the present study we observed that phenol content in the leaves of all mango hybrids, differed significantly ($p \leq 0.01$) under healthy and infested condition caused by mango leafhopper. Accumulation of phenols in Mallika and other hybrids suggests enhancement of phenylpropanoid metabolism and their role in inducing resistance against leaf hopper in mango. The low basal and decreased phenol content in Ratna indicate the susceptibility to leaf hopper and may be attributed to their utilization in scavenging of ROS without concomitant upregulation of phenol synthesis machinery and/or suppression of phenol synthesis as a consequence of oxidative damage to functional biomolecules. The basal levels of phenols weyre maximum in Mallika signifying their defense ability against leafhopper.

The high levels of phenols are directly toxic to insects [43]. Peroxidase and other oxidative enzymes serve to oxidize several of these phenolic compounds in damaged tissues to form reactive quinones [44] which inhibit the further growth of the pathogenic organism by restraining its source of nutrients. Higher constitutive and induced phenolic content in new flush compared to old leaves implied that phenolic content of mango leaves was considerably influenced by maturity. This study substantiated that phenolic content is one of the important factors involved in the resistance-susceptible response of host plant against infestation.

Reducing sugars: High carbohydrate content correlates with high infections in plants [45]. In the present study among the hybrids Ratna recorded highest reducing sugar content trailed by Neeleshan, Nelegoa while Swarna Jehangir and Mallika recorded the least in healthy leaves. The plant sap is the limiting factor for insect's growth, development and survival [46]. According to our results Ratna with excessive levels of sugars offered good nutritional conditions to the leafhopper for its growth and development thus enabling higher infestation rates. Leaf hopper infestation in both new flush and old leaves resulted in maximum decrease in reducing sugar content in Ratna and least in Swarna Jehangir whereas in old leaves Mallika showed least decrease. The primary metabolites are exploited by the organisms for their sustainability and development resulting in decreased reducing sugar content in host tissue. Swarna Jehangir and Mallika with lower reducing sugar content presented incompatible milieu for leafhopper growth thus conferring greater resistance towards infestation. All the hybrids recorded higher quantity of reducing sugar in new flush than old leaves suggesting their greater susceptibility to leaf hopper.

Total chlorophyll: Changes in the chlorophyll content of foliar tissues is an important indicator of disturbed chloroplast development and impaired photosynthetic capacity in plants exposed to a broad spectrum of biotic and abiotic stress. In the current study, leafhopper infestation resulted in decreased chlorophyll content compared to healthy leaves in all the hybrids. Swarna Jehangir, Mallika, Neelgoa and Neeleshan showed moderate decrease in the chlorophyll content while Ratna recorded the maximum decrease suggesting the extent of damage inflicted upon the leaves by leafhopper. The low levels of photosynthetic pigments are caused by most sap sucking insects such as adult leaf hoppers, aphids or thrips which drain sap from the phloem sieve elements of the plants vascular tissue. Heavy infestation leads to chronic shortages of photosynthates and thus severely reduce the photosynthetic potential of the plant [47]. Besides the direct damage, leafhoppers excrete honeydew, which supports the growth of black sooty mold organism (*Capnodium mangiferae*), thus adversely affecting the chlorophyll content and photosynthetic activity of the plant[9]. Old leaves with lower antioxidative capacity suffered greater damage than new flush on leaf hopper infestation as reflected in their lower chlorophyll levels.

Conclusion

The present study revealed that the higher POX, GR, PPO activities and phenolic content played an important role in induced defense mechanism of mango hybrids against leafhopper infestation as observed in Mallika and Swarna Jehangir. Strong induction of POX and PPO enzymes validated the anti-nutritive role of these enzymes in insect defence mechanism. Thus, status of antioxidant enzymes could be a very useful tool in depicting leafhopper infestation resistance of mango. However, further studies on the isoenzymes of the antioxidant enzymes are needed to confirm the genes unique to resistant mango hybrids and thus may provide useful markers for leafhopper resistance.

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