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Deciphering biochemical traits for identifying the yellow vein mosaic disease resistance mechanism in okra

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ABSTRACT

Begomovirus-specific okra yellow vein mosaic virus disease (YVMVD) poses a significant loss to okra yield (~90%). The potent approach to combat this yield loss is the development of resistant cultivars; however, the complex nature of the okra genome poses a challenge. The present study aimed to identify the biochemical basis of YVMVD resistance in okra. We investigated the biochemical alterations in okra introgression lines against YVMVD infection. The maximum disease incidence of 92% was reported in the susceptible group at 45 DOI. Leaf samples collected at pre-flowering, flowering, and post-flowering stages were analysed for superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, hydrogen peroxide, proline, lipid peroxidation, and electrolyte leakage. Independent t-test and ANOVA revealed significant mean biochemical variability between resistant/susceptible groups and infected/healthy groups, indicating that the viral infection caused alterations in the biochemical contents. Except for lipid peroxidation and electrolyte leakage, the biochemical contents/ activities peaked at the flowering stage with major manifestations in resistant genotypes, suggesting their positive relationship with YVMVD resistance in okra. The data obtained herein emphasize the importance of specific biochemicals as crucial markers for identifying the YVMVD-resistant okra varieties.

Key words: *Abelmoschus esculentus*, antioxidants, enzymes, whitefly, YVMVD.

INTRODUCTION

India is the global leader in okra production (10.5mMT) (FAOSTAT, 5). But the high prevalence of biotic stresses, mainly okra yellow vein mosaic virus disease (YVMVD), restricts the crop productivity. The disease is caused by the BYVMV-BYVB complex comprising a mono-/bi-partite genome, and sometimes, it needs an additional beta-satellite molecule to either establish its infection or increase the severity of the symptom manifestation (Venkataravanappa *et al.*, 15). This whitefly-transmitted virus severely affects the yield, with losses ranging from 50% to 90%. Also, early infected plants were found to be more prone to damage as compared to late infection. Therefore, emphasis is now shifting in favour of host plant resistance. Selecting resistance genotypes based on their phenotypes is a tedious process; however, the use of molecular and biochemical markers can expedite this process during the initial growth phase. At the molecular level, characterization in okra breeding is scanty because very few polymorphic markers (Jhanji *et al.*, 8) are available due to highly complex and unstable nature of the okra genome. At the biochemical level, the host-virus interaction is known to cause oxidative damage to plants by triggering increased reactive oxygen species (ROS) production. Excess ROS disturbs the homeostatic defense system of plants, which is activated via antioxidant scavenging action. Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) are the crucial enzymes of this defense system. The SOD catalyzes $O^{2.-}$ to molecular oxygen and hydrogen peroxide, which is converted to water by CAT, POD, and ascorbate peroxidase (APX). Significant changes in the activities of antioxidant enzymes have been previously detected in response to virus infection (Bassiouny *et al.*, 2). Furthermore, the variations in defensive enzymatic activities are not only due to genotypic variations but are also influenced by the various growth stages of the crop. Additionally, ROS-induced cell membrane destruction is another consequence of oxidative damage, which can be measured in the form of electrolyte leakage (EL) and lipid peroxidation (LP).

Previously, several studies have investigated YVMVD severity and crop nutritional quality in okra, but there is a wide lacuna in the stage-specific and temporal responses of biochemicals to virus infection. Thus, it is of utmost importance to unravel this defensive biochemical action at different growth stages of the crop, which may lead to quick and early selection of resistant genotypes.

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MATERIALS AND METHODS

The experiment was conducted for two years (2022 & 2023), both under field conditions (1st year) and in polybags (2nd year) at Vegetable Farms, Department of Vegetable Science, Punjab Agricultural University (PAU), Ludhiana, Punjab. In 1st year, 115 PAUAIOLs from Punjab Padmini × *Abelmoschus angulosus* (BC_1F_7 generation) and 3 cultivated varieties (Punjab-7, Punjab-8, and Pusa Sawani) were sown under standard field conditions in two replications comprising nine plants per row. In 2nd year, the seeds of selected lines were sown under controlled net conditions in polybags (12×12 inches) filled with soil and farmyard manure (1:1). Each line was sown in two replicates with three plants per polybag.

For disease inoculation, the YVMVD inoculum was obtained from fields of vegetable farms and was maintained in an insect-proof net on a susceptible cv. Pusa Sawani. For mass inoculation, healthy test material (on attaining the 2-4 true leaf stage) was exposed to viruliferous whitefly infection for ~15-20 days. Approximately 20 whiteflies and plants were found during the inoculation period. During 1st year, the response of the virus was assessed via visual symptoms at 15, 45, and 75 DOI based on the per cent disease incidence (PDI), disease severity incidence (DSI), severity grade (0-4), and response factor (0-1) (Bag *et al.,* 1). During the second year, 19 PAUAIOLs (representing each response category), along with three cultivated varieties (Punjab-7, Punjab-8, and Pusa Sawani) were selected based on the CI value obtained during 1st year and again sown and screened for PDI, DSI, and CI and were evaluated for biochemical estimations in two sets. One set was control, *i.e*., without inoculation, and the other was with inoculation (treatment). From both the sets, sampling was done at pre-flowering, flowering, and post-flowering (*i.e*., at 15th, 45th and 75th DOI), and were stored at -30˚C for biochemical analysis.

The biochemical estimations were performed in the Department of Biochemistry, PAU, Ludhiana, using standardized procedures. Lipid peroxidation was determined in terms of malondialdehyde (MDA) content, formed by its reaction with thiobarbituric acid. Absorbance was determined at 532 and 600 nm wavelengths (Dhindsa *et al.,* 4). Liu & Huang (10) method was used to assess EL. The conductivity of the solution was measured both before and after boiling the samples at 100 ˚C in water bath for half an hour. The electrolyte leakage was calculated using the formula: Conductivity (before boiling) (Conductivity after boiling)*100. The standardized methods of Giannopolitis and Ries (7), Sinha (14), Garcia-Limones *et al.* (6), Shannon *et al.* (12), and Nakano and Asada (11) were followed for the estimations of SOD, H_2O_2, CAT, POD, and APX, respectively. The extract was prepared *via* homogenizing leaf samples (0.2 g) with ice-cold sodium phosphate buffer (2 ml-100 mM, 2 ml-100 mM, pH 7.5), followed by centrifugation at 10,000 g (4 ºC) for 10 minutes. The supernatant obtained was used as a common enzyme extract. For SOD, the enzyme assay (3 ml) comprised of sodium phosphate buffer (2.9 ml-100 mM, pH 7.0) and enzyme extract (0.1 ml). The internal control was prepared similarly, but without enzyme extract and under illumination; however, the blank contained enzyme extract and was kept in the dark. After sample exposure to fluorescent light (120 W) for 15 minutes, and to dark for 10 minutes, the absorbance was recorded at 560 nm. For H_2O_2 content, supernatant (1 ml) was mixed with 2 ml mixture of 5% potassium dichromate and glacial acetic acid (1:3). The absorbance was measured at 570 nm, and the standard curve was prepared using H_2O_2 in the range of 20-100 µmoles. For catalase, supernatant (0.1 ml) was mixed with 1.9 ml assay buffer (50 mM sodium phosphate buffer-pH 7.5) and 30 mM hydrogen peroxide. The change in absorbance was recorded for 3 minutes after every 30 sec. at 240 nm. For POD, enzyme extract (0.1 ml) was added to guaiacol (3 ml-50 mM) and H_2O_2 (0.1 ml-30 mM). The increase in absorbance was recorded at an interval of 30 sec. for 3 min at 470 nm. For APX estimation, sodium phosphate assay buffer (1 ml- 50 mM, pH 7.5) was mixed with ascorbic acid (0.8 ml-0.5 mM), enzyme extract (0.2 ml), and H_2O_2 (1 ml-30 mM). Every 30 seconds for 3 minutes, the absorbance was measured at 290 nm. To determine proline content, leaf tissue (0.1 g) was homogenized with sulphosalicylic acid (2 ml), and centrifuged at 10,000 g for 10 minutes. The assay mixture consisted of enzyme extract (1 ml), glacial acetic acid (2 ml-3%), and ninhydrin reagent (2 ml). The mixture was transferred to an ice bath after keeping it for one hour at 100°C. To this mixture, toluene (4 ml) was added and mixed, and the toluene layer at the top was collected to record absorbance at 520 nm against a blank (toluene) (Chinard, 3). Using proline in the range of 10-50 µg, the proline concentration was determined.

The data (mean ± S.E. of four replicates) were statistically analyzed by using an independent t-test and ANOVA (*P≤0.05*) to compare the means of inoculated/uninoculated material and resistant/susceptible groups. Tukey's test was used to determine the significance of differences. The Pearson correlation coefficient was determined at the 1% and 5% level of significance using SPSS 20.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

The inoculated test material revealed severe yellowing and chlorosis in leaf veins, along with shorter plant height as compared to uninoculated material. The PDI in okra leaves progressed from 15 to 75 DOI (Fig. 1a & b) with 20-72.2% in year 1 and 20-92.3% in year 2. The maximum PDI was recorded at 45 DOI during both years in the susceptible group, indicating extensive disease development. There were no disease symptoms on highly resistant lines until maturation. The results obtained are synchronous with Khade *et al.* (9), thus validating our study.

Based on CI in 1st year, 111 lines were found to be highly resistant (HR), 5 PAUAIOLs, *i.e.*, 302, 318, 429, Punjab-7 and Punjab-8 were moderately susceptible (MS), and PAUAIOL-336 and Pusa Sawani as susceptible (S) and highly susceptible (HS), respectively. From the HS category (111), randomly 15 lines were selected, which were screened for PDI in the 2nd year, along with five MS and one S, and one HS line. The material was then again grouped into HS, S, MS, and HR categories on the basis of CI. The results obtained were reproducible in the second year, demonstrating that the response to YVMVD infection was genotype-dependent.

For the studied biochemicals, *viz.,* LP, EL, SOD, H_2O_2, CAT, POD, APX, and proline, analysis of variance revealed significant variability at the 5% level of significance across all the stages of infection, across all the introgression lines and cultivated varieties, and under both treatments. The independent t-test revealed significant variability amongst means of inoculated/uninoculated and resistant/ susceptible groups of all studied biochemicals.

The mean LP content in inoculated samples was increased by 1.32-fold from 15th to 45 DOI in both resistant and susceptible material, though it was higher in susceptible ones (Fig. 2a), indicating a more severe membrane degradation during flowering. At post-flowering, LP level declined for both control and treated material, though it was higher in treatment, suggesting a consistent but declining response. The results are synchronous with previous studies of Bassiouny *et al.* (2) and Singh *et al.* (13) on infected okra and tomato, respectively. Amongst susceptible ones, the maximum lipid peroxidation was harboured by Pusa Sawani (83.86 nmoles/mg FW). More MDA in susceptible group could be explained as the defense machinery would be more prominent in removing the ROS in the resistant group than susceptible ones. The susceptible group showed higher EL with a maximum in Pusa Sawani (93.5 %), followed by PAUAIOL-336 (92.33%). Among stages, a significant increase in EL was observed from 15th to 45 DOI in both resistant and susceptible groups (Fig. 2b). Further, LP and EL revealed a positive relationship with PDI, explaining more severe membrane degradation and thence more electrolyte leakage in diseased tissues.

The SOD activity was significantly increased in both resistant and susceptible groups from pre-flowering (273.83 & 203.79 Units/min/g FW tissue) to flowering (328.65 & 278.78 Units/min/g FW tissue), followed by an abrupt decrease at post-flowering (212.37 & 150.37 Units/min/g FW tissue), respectively (Fig. 2c). Previously, Bassiouny *et al.* (2015), (2), and Singh *et al.* (13) reported an increase in SOD enzyme activity after inoculation. Concerning H_2O_2 content, from pre-flowering to flowering, the average H_2O_2 content of inoculated resistant PAUAIOLs significantly increased by 47.79%, while the corresponding figure in inoculated susceptible material was 34.21% (Fig. 2d). Earlier findings of Singh *et al.* (13) in BYVMV-infected okra support our results of a 48% surge in average H_2O_2 content in inoculated resistant PAUAIOLs from pre-flowering to flowering. For catalase (Fig. 2e), resistant PAUAIOL-325 on inoculation exhibited the highest activity at flowering, whereas Pusa Sawani had the minimum

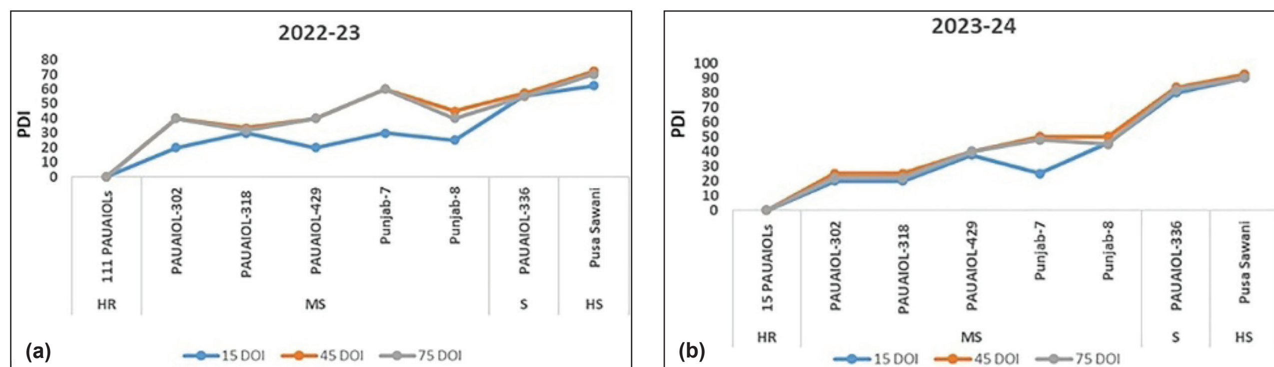

Fig. 1. Per cent disease incidence (PDI) in okra PAUAIOLs and cultivated varieties (a) 2022-23 and (b) 2023-2024.

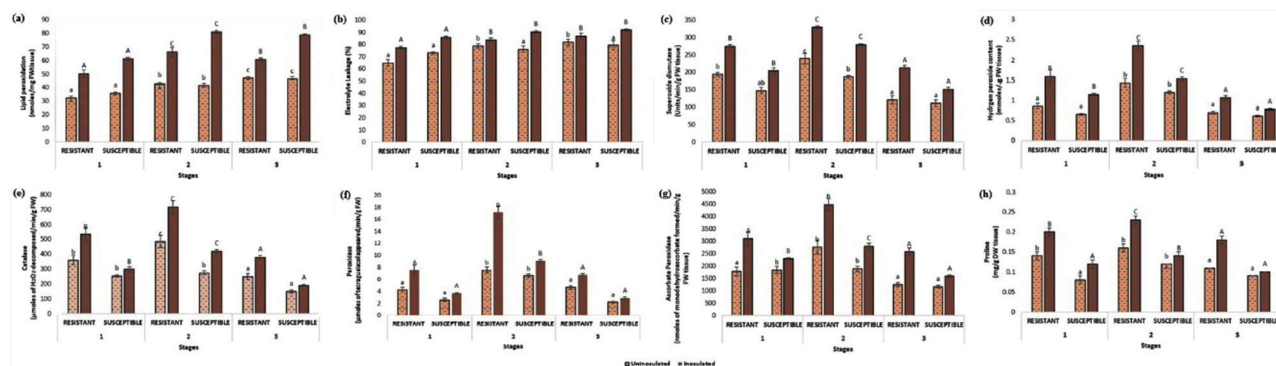

Fig. 2. Biochemical variability in plants of various resistant and susceptible PAUAIOLs and cultivated varieties against YVMV disease. a) Lipid Peroxidation (LP); b) Electrolyte Leakage (EL); c) Superoxide Dismutase (SOD); d) Hydrogen Peroxide (H_2O_2); e) Catalase (CAT); f) Peroxidase (POD); g) Ascorbate Peroxidase (APX); h) Proline. Error bars depict average ± standard error values; the same letters on bars represent non-significance at the 5% level of significance; small (a, b, c) and capital letters (A, B, C) show significant differences among uninoculated and inoculated material, respectively.

(892.29 and 385.47 µmoles of H_2O_2 decomposed/ min/g FW). In our study, from pre-flowering to post-flowering, 35% higher catalase enzyme activity was recorded in inoculated-resistant PAUAIOLs, which is comparable to the previous results of Bassiouny *et al*. (2). For POD, resistant lines showed the highest activity (17.10 µmoles of tetraguaicol formed/min/g FW tissue), reflecting enhanced stress responses, whereas susceptible lines showed consistently lower POD activity (Fig. 2f). Ascorbate peroxidase activity showed significant variability under both treatments and across the stages, as well as across genotypes. In the inoculated set, maximum APX activity was achieved at flowering, with an increase of 44.39% in the resistant group, whereas in the susceptible group, the value observed was almost half, *i.e.*, 22.47% (Fig. 2g). At post-flowering, the APX activity declined, though values were higher in inoculated material. At flowering, PAUAIOL-301 (HR) harbored the highest proline content (0.29 mg/g DW tissue), indicating a strong defense response during peak infection, whereas, PAUAIOL-306 (0.19 mg/g DW tissue) had the minimum content among the resistant inoculated PAUAIOLs. However, it was further reduced to 0.12 millimoles/mg DW tissue in susceptible inoculated PAUAIOL-336 (S), reflecting a weaker defense response (Fig. 2h). In summary, inoculation significantly increased both enzymatic and non-enzymatic antioxidants during the flowering stage, making it crucial for identifying the maximum YVMVD infection.

To determine the relationship between different biochemicals and PDI, a correlation matrix was generated (Table 1a-c). It was observed that there was a negative and significant association ($P \leq 0.01$ and $P \leq 0.05$) with PDI at all three stages except LP and EL. This information may help in identifying reliable markers for resistance and may reduce dependence on the visual scoring alone. At pre-flowering (Table 2a), strong inverse correlations were observed between PDI and SOD, suggesting strong enzymatic action during the initial phase of infection, leading to a diminished disease response. At flowering (Table 2b), stronger and more significant associations were observed with higher r-values of proline, CAT, APX, and H_2O_2 (r = -0.797, -0.663, -0.603, -0.688), respectively, reflecting maximum defensive action at this stage. At post-flowering (Table 2c), there was a decrease in r-values, but the relationships were significant. These results validate the role of enzymatic and non-enzymatic antioxidants in acquiring resistance against YVMVD infection. Further, all the enzymatic antioxidants are positively related to each other, which indicates their coherent action in dealing with stress.

Conclusively, the study provides compelling evidence that virus infection in okra resulted in higher expression of biochemicals as compared to control plants, which suggests their defensive response in the plants in aid to protect against the viral attack. With time progression, the enzymatic and non-enzymatic antioxidant activities/contents were found to be increased with prominence at flowering; thereafter, the expressions were reduced. Moreover, higher contents/activities of biochemicals in resistant lines indicate that they are positively correlated to YVMVD resistance in okra, thus concluding SOD, H_2O_2, CAT, POD, APX, and proline as the crucial biochemical markers for identifying host plant-virus resistance reactions to YVMVD in okra. Elaborative gene-level studies of these biochemicals, followed by their functional validation

Table 1: Correlation matrix between per cent disease incidence (PDI) and various biochemical attributes at a) 15th DOI (Pre-flowering), b) 45th DOI (flowering), and c) 75th DOI (post-flowering).

(a)	LP	EL	Proline	SOD	H_2O_2	CAT	POD	APX	PDI
LP	1	.419**	-.491**	-.665**	-.202	-.537**	-.484**	-.181	.567**
EL		1	-.482**	-.553**	-.298**	-.495**	-.592**	-.384**	.700**
Proline			1	.694**	.237*	.622**	.516**	.536**	-.659**
SOD				1	.298**	.658**	.514**	.505**	-.759**
H_2O_2					1	.216*	.141	.350**	-.433**
CAT						1	.566**	.450**	-.582**
POD							1	.340**	-.527**
APX								1	-.516**
PDI									1
(b)	LP	EL	Proline	SOD	H_2O_2	CAT	POD	APX	PDI
LP	1	.217*	-.666**	-.582**	-.427**	-.426**	-.515**	-.494**	.606**
EL		1	-.432**	-.523**	-.580**	-.550**	-.143	-.480**	.522**
Proline			1	.667**	.586**	.605**	.513**	.652**	-.797**
SOD				1	.634**	.623**	.525**	.405**	-.692**
H_2O_2					1	.728**	.450**	.482**	-.688**
CAT						1	.404**	.609**	-.663**
POD							1	.339**	-.511**
APX								1	-.603**
PDI									1
(c)	LP	EL	Proline	SOD	H_2O_2	CAT	POD	APX	PDI
LP	1	.272*	-.725**	-.563**	-.447**	-.685**	-.730**	-.551**	.730**
EL		1	-.270*	-.235*	-.464**	-.415**	-.400**	-.242*	.370**
Proline			1	.563**	.382**	.515**	.713**	.590**	-.726**
SOD				1	.647**	.623**	.650**	.351**	-.651**
H_2O_2					1	.518**	.646**	.198	-.588**
CAT						1	.655**	.577**	-.703**
POD							1	.328**	-.783**
APX								1	-.573**
PDI									1

*. P≤ 0.05 level. **. P≤ 0.01 level.

LP - Lipid peroxidation, EL - Electrolyte leakage, SOD - Superoxide dismutase, H_2O_2 - Hydrogen peroxide, CAT - Catalase, APX - Ascorbate peroxidase, POD - Peroxidase

and expression analysis, could further reinforce these outcomes. Conclusively, the present study reveals that YVMVD resistance mechanisms involve biochemical defense systems, and data obtained herein could be manipulated for developing YVMVD-resistant okra germplasm.

AUTHOR'S CONTRIBUTION

Conceptualization of research (SG); Designing of the experiments (SG and MP); Contribution of experimental materials (MP, AS and MKS); Execution of field/lab experiments and data collection (SG and NS) Analysis of data and interpretation (SG and NS); Preparation of the manuscript (SG and NS)

DECLARATION

The authors declare no conflict of interest.

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