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Biochemical and Molecular Variations of Guaiacol Peroxidase and Total Phenols in Bacterial Wilt Pathogenesis of *Solanum melongena*

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Abstract

Plants respond to bacterial pathogen attack by activating various defense responses, which are associated with the accumulation of several factors like defense-related enzymes and inhibitors which serve to prevent pathogen infection. The present study focused on the role of the defense-related enzyme and gene expression of Guaiacol preroxidase and Total phenol content in imparting activities was analyzed by selecting three different eggplant cultivars against bacterial wilt pathogen *Ralstonia solanacearum*. The temporal pattern of induction of these enzymes showed (42.72 U) maximum activity at 21 h after the pathogen inoculation (hpi) in resistant cultivars. The expressions of defense genes increased 5.5 folds in resistant eggplant cultivars after pathogen inoculation. The total phenol content increased significantly ($P<0.05$) in resistant cultivars upon pathogen inoculation compared to susceptible and highly susceptible cultivars. The biochemical and molecular markers provided an insight to understand the first line of defense responses in eggplant cultivars upon inoculation with the pathogen.

Keywords: *Ralstonia solanacearum*; Guaiacol peroxidase; Bacterial wilt; Eggplant; QRT-PCR

Introduction

Brinjal (*Solanum melongena* L.), also known as Aubergine or the Eggplant, it is a commonly cultivated vegetable in countries like China, India, Egypt and Iran. Some of the common diseases affecting the production of eggplant are Damping off, *Phomopsis* blight, *Verticillum* wilt, Bacterial wilt, *Cercospora* leaf spot, and Little leaf of eggplant. One of the most common and devastating disease that affects the production of eggplant is the Bacterial wilt caused by *Ralstonia solanacearum*. It is gram-negative, rod-shaped, strictly aerobic bacterium which is primarily a soil-borne and waterborne pathogen [1]. It infects host plants from their roots, infected from damaged wounds formed by lateral root emergence or by root damage caused by soil borne organisms. *R. solanacearum* infect the roots or stems, they colonize the plant through the xylem in the vascular bundles. Wilting of the whole plant may follow rapidly if environmental conditions are favorable for the pathogen. Plant-to-plant infection can occur when bacteria shed from infected roots move to roots of nearby healthy plants. Therefore, the management of this disease is very difficult [2-4].

When a pathogen invades a plant, it defends itself by means of active and passive defense mechanisms. The passive or pre-existing defense mechanisms include structural barriers which prevent the colonization of the tissue. The active defense mechanisms involve the generation of hypersensitive response (HR), the production of phytoalexins and pathogenesis-related proteins, the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (termed as oxidative bursts), lignifications, and reinforcement of cell wall structural proteins and deposition of callose. The efficacy of these defense responses together with anti-oxidant responses often determines whether the plants are susceptible or resistant to pathogen infection.

Guaiacol preroxidase (GPOD: EC.1.11.1.7) are prevalent enzymes that participate in a fundamental role of plant defense mechanisms. GPOD is a heme containing glycosylated proteins that catalyze the oxido-reduction of aromatic electron donor such as guaiacol and pyragallol at the cost of hydrogen peroxidase. GPOD enzymes structurally composites of four conserved disulfide bridges and contain two structural Ca^{2+} ions interconnected [5]. Higher plants possess a number of different GPOD isoenzymes in plant tissues localized in vacuoles, cytosol and cell wall [6,7]. GPOD also called as GPX, POX are widely accepted as stress "enzyme" GPOD can function as effective quencher of reactive intermediary forms of Oxygen and peroxy radicals under plant pathogen interactions. GPOD have been implicated in many significant mechanisms was revived, In conclusion several reports explained GPOD play a fundamental roles such as lignification of cell wall, oxidation of hydroxy-cinnamyl alcohol into free radical intermediates, degradation of IAA, regulation of plant cell elongation, biosynthesis of ethylene, wound healing, and polysaccharide cross linking [8].

Various plants defense mechanisms were studied against abiotic and biotic stresses conditions of the environment by induce the activity of GPOD. The increase in GPOD activity during resistance in different hosts against different pathogens has been demonstrated. Southerton and Deverall [9] reported that increases in GPOD activity were greater during the expression of resistance in wheat against the leaf-rust fungus than in susceptible plants. Increase in the GPOD activity was observed during the interaction between rice and *Xanthomonas oryzae pv. Oryzae* [10-12] and between cotton and *Xanthomonas axonopodis pv. Malvacearum* [13]. Guaiacol peroxidase might also play an important role in *Cassava* bacterial blight caused by *Xanthomonas axonopodis pv. Manihotis* [14,15]. Ikegawa, et al. [16] showed that the activity of ionically bound cell wall peroxidases was significantly higher in incompatible interactions of oat with *Puccinia coronata f.* sp. *avenae* than in a compatible one. Mohammadi and Kazemi [17] reported that there is an increased GPOD activity in heads of both

resistant and susceptible wheat cultivars during various developmental stages following the inoculation with *Fusarium graminearum*. Peroxidase activity was increased in resistant pearl millet cultivars infected with *Sclerospora graminicola* than in susceptible cultivars [18]. Delannoy, et al. [19] suggests that cotton peroxidases may have various functions in the defense response to *Xanthomonas* infections. Mlickova et al. [20] showed that increased GPOD activity occurred in two stages in *Lycopersicon* species resistant to *Oidium neolycopersici.* Silva et al. [21] revealed the increased GPOD activity in relation to host cell wall lignifications in coffee resistance to orange rust.

Up-regulation of peroxidases (membrane-bound class III peroxidases) in maize seedlings stress responses activated by methyl jasmonate and salicylic acid pathways reviled that, GPOD not only function in abiotic stress but also in pathogen response [22]. Mandal et al. [23] undertook to investigate the biochemical aspects of oxidative burst, anti-oxidative mechanism and cell wall reinforcement as initial defense responses of two tomato cultivars viz., Arka Meghali (susceptible) and BT-10 (resistant) against the devastating bacterial wilt pathogen *R. solanacearum*. The tomato variety BT-10 has been identified as resistant to *R. solanacearum* due to rapid increase in GPOD activity. Meanwhile in our previous reports Vanitha and Umesha [24] analyzed Pre-treatment of tomato plants with *P. fluorescens* triggered the increased preroxidase. Plant growth promoting rhizobacteria (PGPR) microorganisms were assessing their capacity to prime rice seedlings against stress challenge (salt and *Xanthomonas campestris* infection). GPOD was significantly primed by both strains 24 h after stress challenge was reported by Cristobal et al. [25]. The involvement of GPOD in defense mechanism of *R. solanacearum* infection in eggplant remains unclear. In order to accomplish this void information, the present study was undertaken to analyze role of guaiacol peroxidase and total phenol content in host resistance of bacterial wilt.

Material and Methods

Bacterial source and growth conditions

Ralstonia solanacearum was isolated from soil source in the agricultural fields of Karnataka state, India. The bacteria were isolated and maintained on a semi-selective Kelman's TTC medium [26]. The isolated pathogen was confirmed by biochemical/physiological, pathogenicity tests. Biochemical characterization of *R. solanacearum* was performed based on biochemical tests: Gram staining, KOH solubility, starch hydrolysis test [27], lipase activity, Kovacs' oxidase test [28], gelatin hydrolysis and oxidative/fermentative metabolism of glucose. Strains were classified to biovar using a variation in physiological test developed by Hayward [29] in a test tube, the determination of biovars of *R. solanacearum* is based on utilization of Hayward medium (Dextrose, lactose, dulcitol, or D-mannitol) along with D-ribose and meso-inosital were used to separate biovars 2A and 2B by inoculating 20 μl of *R. solanacearum* culture at concentration of (1x108 cfu ml-) and tubes were incubated at 28°C for 15 days. Uncultured tubes were kept as control. Molecular identification methods were performed by PCR amplification using 16S rRNA [30]. The glycerol stocks of bacterial cultures were prepared and submitted to the Stock Collection Centre (Department of Studies in Biotechnology, University of Mysore, Mysore, India) and were used for all further inoculations.

Chemicals and collection of seed samples

All chemicals used in sample preparation were of analytical grade. All authentic standards and primers were procured from Sigma–Aldrich Chemical Co. Ltd (Bangalore, India). Molecular biology chemicals were procured from Genetix, Bangalore, India. Seeds of eggplant cultivar Arka Shirish (resistant to bacterial wilt) were procured from National Seed Corporation, Bangalore, India. MEBH-11 (susceptible) and Chaman 363 (highly susceptible) cultivars seeds obtained from Annadatha Kendra and private seed traders respectively of Mysore, India. They were disinfected by surface sterilization with 3% (v/v) sodium hypochlorite solution for 5 min and washed with sterile distilled water thrice.

Ralstonia solanacearum inoculation to eggplants

The seeds were sown in 9 cm diameter plastic pots under greenhouse conditions. Eggplant cultivars (Arka Shirish, MEBH-11 and Chaman 363) were subjected to screening against bacterial wilt disease by following standard procedures of Trans-Kersten et al. [31]. Briefly, seedlings were raised from all the cultivars of eggplant in 9 cm diameter plastic pots containing a mixture of sterilized soil, sand, coir pith and farmyard manure in the ratio of 2:1:1:1. For each cultivar, 16 plants in three replicates were maintained under greenhouse conditions at the temperature of 28 ± 2°C and the experiments were repeated thrice. The *R. solanacearum* culture grown on the Kelman's TTC medium was suspended in distilled water and centrifuged at 12000 rpm for 10 min and the pellet obtained was re-suspended in sterile distilled water. The density of cell suspension was adjusted to 1x108 cfu/ml using spectrophotometer (Beckman Coulter, California, USA). The bacterial suspension (15 ml) was poured directly near the root system of 24-day-old eggplant seedlings. Control plants were mock inoculated using sterile distilled water. The seedlings were harvested at time intervals of 0, 3, 6, 9, 12, 15, 18, 24, up to 120 h, that is with an interval of 3 h each after pathogen inoculation (HPI). The roots were washed in tap water and immediately frozen by storing in deep freezer at -80°C for further analysis. The experiments consisted of three replicates and were repeated three times. Eggplant seedlings were examined carefully for the typical bacterial wilt symptoms such as stunting, wilting, vascular browning and often rapid death from the day of inoculation and up to 45 days.

Enzyme assay and native-PAGE analysis of GPOD

Guaiacol peroxidase (EC.1.11.1.7): Guaiacol peroxidase assay was performed by following the standard procedure specified by Naveen, et al. [32]. The reaction mixture consisted of 2.9 ml of substrate buffer (125 ml of 0.05 M Guaiacol, 153 ml of 30% H_2O_2 [v/v] in 50 ml of 0.1 M potassium phosphate buffer [pH 7.0]) and 0.1 ml of enzyme extract. Increase in absorbance was (470 nm) recorded for 1 min and expressed as the change in the absorbance at 470 nm min/mg/protein. GPOD activity was expressed as units of enzyme activity (U). Units were calculated using a molar extinction coefficient of 26.6 units for tetra guaiacol. The concentration of protein in the enzyme extracts was determined by standard procedure of Bradford (1976), using BSA (Sigma, USA) as standard.

The isoforms profile of GPOD was examined by Native-PAGE (Native polyacrylamide gel electrophoresis) following the standard protocol of Laemmli [33]. GPOD enzyme extracted (100 mg protein) from resistant, susceptible and highly susceptible eggplant cultivars at 18 h. Three cultivars inoculated with pathogen and controls were

loaded on top of 8% (w/v) polyacrylamide gels in a vertical gel electrophoresis (Tarsons, Bangalore, India). Electrophoresis gels were run in a voltage of 50 V initially for 1 h and further complete electrophoresis was performed in 100 V. The electrophoresis was run with Tris-base buffer (6.0 g Tris-base, 14.4 g glycine dissolved in 1 L of distilled water). After electrophoresis, GPOD isoforms were stained with 100 mg benzidine/ml of absolute alcohol by soaking the gels in staining solution and made up to 40 ml using distilled water. Clear solution was obtained by adding 100 µl of glacial acetic acid, H_2O_2 (50 µl) was added to the filtered staining solution at the end and gels were incubated in the solution till bands appear and gels were documented using Geldoc 1000 System-PC (Bio-Rad, Gurgaon, India).

RT-PCR studies

Total RNA was extracted from seedlings of R (Arka Shirish), S (MEBH-11) and HS (Chaman 363) eggplant cultivars by grinding them into powder in pre-chilled mortar and pestle with liquid nitrogen. The powder (100 mg) was homogenized and centrifuged at 12, 000 rpm for 10 min in 1 ml of TRIzol@ reagent (Sigma, Bangalore, India). The supernatant was again centrifuged in 200 µl of chloroform and treated with 1 ml of isoamyl alcohol and finally RNA was precipitated with ethanol [34]. cDNA was synthesized by reverse transcription reaction by following the manufacturer's instruction using cDNA synthesis kit (Thermo Scientific, Bangalore, India).

The primers used in the present studies were designed using the complete cDNA sequences of GPOD sequences reported in the GenBank. Sequences were retrieved from the nucleotide database of NCBI of solanaceae crops (tomato, potato, eggplant). The sequences were aligned using CLUSTAL W software and specific sets of primers were designed (Table 1) using Primer 3 software; the designed primers were custom synthesized from Sigma (Bangalore, India). Semi-quantitative RT-PCR was performed in 25 µl total volume of reaction mixture, containing 2 µl of synthesized cDNA, 1 U of Taq DNA polymerase and 1 µl of 100 mM dNTPs, 2.5 µl of 10 x buffers and 100 pmol of primers. PCR was carried out in a thermocycler (Labnet, Multigene gradient, California, USA) under the following conditions: Initial denaturation at 94°C for 5 min followed by 18-25 cycles at 94°C for 1min, annealing at 56°C for 1 min, 72°C for 1 min, the final extension at 72°C for 10 min.

Gene	Encoding enzyme	Primers pair Sequence (5'-3')	Annealing Temperature	GenBank accession
GPOD	Guicol peroxidase	F: ACCGTGAGCGAGGACTACCT R: AGCGTCAAGTGAGCCTTAGC	56.0°C	KJ748198
β-actin	Positive control	F: TGGTCGGAATGGGACAGAAG R: CTCAGTCAGGAGAACAGGGT	60.0°C	--
Primed primer sets were designed by primer 3 software and annealing temperature used to amplify GPOD, along with their Gen Bank accession number. β-actin were synthesized from previous reports.				

Table 1: Primers used for RT-PCR assays along with their accession numbers.

Amplified PCR products were purified using QIA quick gel extraction kit (Qiagen, Hilden, Germany) by following the manufacturer's instructions. Amplified products of GPOD were cloned into a pGEM-T Easy vector adding T4 DNA ligase and 1x ligation buffer in 30 µl volume. Transformation was carried out using *E. coli* JM109 by heat shock method. The competent cells were placed on LB plates containing ampicillin incubated overnight at 37°C. The white transformed clones were confirmed by colony PCR with the same primers. The plasmid DNA was isolated and sequenced commercially (Eurofins, Bangalore, India). The amplified sequence data of enzymes was analyzed by BLAST sequence similarity search and the phylogeny of all genes were constructed with sequence availability in NCBI database.

Total phenol estimation

Fifteen different eggplant cultivars of seedlings (each 0.5 g) were homogenized using 80% methanol (w/v) and centrifuged at 10,000 rpm for 10 min. The supernatant was air dried and dissolved in 5 ml of distilled water. The total phenolic content was determined using Folin-Cioculteou reagent as described by Vanitha, et al. [35] using a standard curve of known concentrations of Gallic acid. The phenolic content was expressed as µg phenol g^{-1}.

Statistical analysis

Green house experiments and enzyme activities were performed in four replicates and repeated thrice. Data were analyzed by two-way ANOVA (treated and control) using SPSS software. The means were compared for significant effects on enzyme activity by pathogen inoculation by the magnitude of the F-Value ($P \leq 0.05$).

Results and Discussion

Bacterial source and identification

The bacterial wilt causing pathogen *R. solanacearum* in eggplants were isolated and characterized from infected fields [36]. Isolates of *R. solanacearum* from soil, plant material and seeds were cultured using semi selective media and typical mucoid creamy white colonies with pink centers were observed as reported earlier [23-28] Further different isolates of *R. solanacearum* were further subjected to biochemical/physiological assays along with hypersensitive and pathogenicity tests [30,36,37]. These pathogens stained pink red for Gram's reaction and thin viscid mucoid strand for KOH solubility indicating gram negative nature. *R. solanacearum* indicated negative for gelatin hydrolysis, starch hydrolysis test as well as lipase activity. Kovac's oxidase tests showed a positive result by immediate colour change to blue by *R. solanacearum*. The pathogen changed the color from green to yellow indicating a positive for oxidation test, whereas in

the fermentation test these pathogens did not shown any reaction. Susceptible tomato and eggplant cultivars when inoculated with *R. solanacearum* isolated from the soil, seed and plant material showed bacterial wilt symptoms such as vascular browning, stunting, wilting and often rapid death of eggplants. Control plants did not show similar disease symptoms. Yellowing of leaf followed by necrosis was evident in tobacco plants within 48 h of infiltration with both bacterial isolates, whereas control leaves did not show any change in leaf morphology. Biochemical/physiological tests, hypersensitivity and pathogenicity tests were used in the identification and confirmation of the isolated pathogens as *R. solanacearum*. The identity of the phytobacterium was confirmed by amplification of 16S rRNA. The *R. solanacearum* isolate has been deposited in the Department Stock Collection Centre (Department of Studies in Biotechnology, University of Mysore) and designated as *R. solanacearum* DOB R1. The amplified sequence of all three regions of phytobacterium has been submitted to NCBI Genbank database with accession number KF021616 [30].

GPOD activity

The temporal changes of GPOD enzyme activity in R cultivar (Arka Shirish) was gradually increased upon pathogen inoculation and reached its peak at 21 hpi. In R cultivar the GPOD enzyme activity was 10.59 U in uninoculated control, which was significantly increased to 42.72 U at 21 hpi upon pathogen inoculation (Figure 1). Meanwhile, the GPOD activity in S cultivar MEBH-11 was 8.9 U in control plants which is gradually increased to 31.52 upon pathogen inoculation. Whereas, in HS cultivar Green Round, the GPOD enzyme activity was 0.4 U in uninoculated control which was increased to 1.5 U hours after pathogen inoculation. Although the activity of antioxidative enzyme GPOD was increased after infection of *R. solanacearum*, the activity of enzyme was rapidly increased in R cultivar Arka Shirish followed by S cultivar MEBH-11 and HS cultivars Green Round in early time intervals of challenge inoculated. Defense-related enzymes GPOD, PPO, PAL, and LOX are known to play a major role biosynthesis of cell wall strengthening material and secretion of antimicrobial compounds such as phytoalexins, furanocoumarin, quinones, and pterocarpan [21] which determine the host resistance. Similar results were exhibited when Cerebroside elicitors initiate accumulation of H_2O_2 followed by the production of plant defense-related enzymes viz., PAL, POX, PPO, and LOX in *Capsicum annuum L.* Similarly, Increase in the GPOD activity was observed during the interaction between tomato and *R. solanacearum*, rice and *Xanthomonas oryzae pv. oryzae, cotton and Xanthomonas axonopodis pv. malvacearum* Cassava bacterial blight caused by *Xanthomonas axonopodis pv. manihotis* [7-13]. Whereas, similar results were observed in the present study that GPOD play a major role in activation of defense against *R. solanacearum*. A total of three isoforms of GPOD were expressed and its intensity varied between cultivars and even between control and inoculated seedlings. The GPOD isoforms were faint in S and HS seedlings when compared to R seedlings. There was no difference in number of isoforms in all the three categories of seedlings used which correlated the results of Laemmli and Vanitha, et al. [30,32].

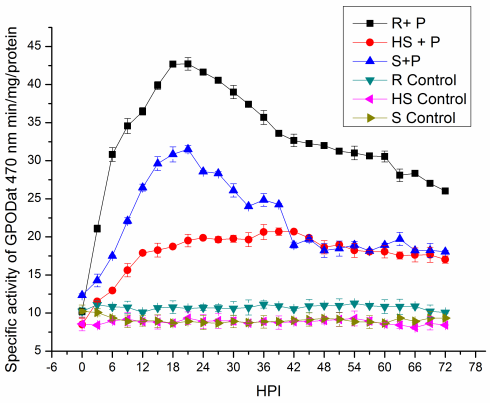

Figure 1: Temporal study pattern of GPOD activity in Resistant (R), Susceptible (S) and Highly Susceptible (HS) eggplant cultivars, Eggplant seedlings were raised from followed by challenge with pathogen. Both treated and control seedlings were harvested at different hours after pathogen inoculation (HPI), and subjected to GPOD enzymatic estimation. The data is expressed as the average of three independent experiments with three replicates. Bars indicate standard errors.

Peroxidase catalyzes the last step in the biosynthesis of lignin and other oxidative phenols. Seed treatment with Lactic acid bacterium (LAB) induced the defense related activities of GPOD. They indicated a significant increase in GPOD activity at 24 hpi, observed in the LAB treated seedlings challenge inoculated with *R. solanacearum* suggesting the induction of resistance in tomato plants. The control seedlings reported lowest GPOD activity with or without pathogen infection. However, the seedlings inoculated with Lactic acid bacterium isolate alone also exhibited high GPOD activity in comparison with the control. Similarly, the screened resistant cultivars of eggplant showed similar results when compared to induced resistance in cultivars of tomato against bacterial wilt [38].

RT-PCR studies

Peroxidase is a defense-related enzymatic gene, with broad spectrum activity and it plays key roles in plant-pathogen interactions. GPOD gene is believed to be one of the most significant factors of the plant's biochemical defense against pathogenic microorganisms, and is actively involved in self-regulation of plant metabolism after infection. Based on the temporal pattern activity of the time intervals for GPOD activity was found to be 18 hpi for both resistant and highly susceptible eggplant cultivars. In GPOD, the gene expression was high in R cultivars followed by S cultivars and the mRNA transcript accumulation was least in HS cultivars. In order to investigate the role and fold changes of defense genes in protection of ROS caused by bacterial wilt, we have analyzed the activity of GPOD. The GPOD gene expression was significantly increased in R cultivar (Arka Shirish) at 18 hpi and was significantly up-regulated to 5.5 folds upon pathogen inoculation when compared to control (Figure 2) whereas, in S and HS eggplant cultivars, the GPOD gene expression pattern was down-regulated to the extent of 0.9 and -3.2 respectively compared to control. A partial amplified cDNA from the eggplant was cloned and

sequenced commercially, which was later compared with NCBI database using BLASTN algorithm. The GPOD sequences represented homology as cytosolic GPOD isoforms, in which the genes show 100% homology with GPOD of *S. melongena*. *S. tuberosum* exhibited 96% and 98% homology for GPOD respectively, whereas 95% and 100% homology in *S. lycopersicum*, but less than 90% was showed with respect to *C. annuum* and *N. tabacum*. The phylogenetic tree constructed using the deduced nucleotide sequences of all the four genes with different genera of *Solanaceae* species in which genes are conserved across all plant species with slight evolutionary differences between them. The sequences were deposited in the GenBank database of NCBI Accession numbers KX347437.

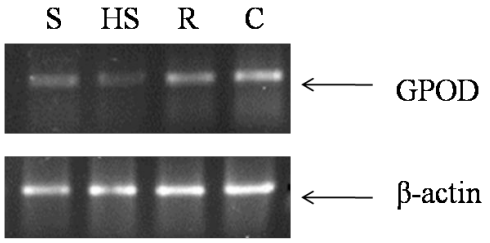

Figure 2: Induced gene expression patterns of defense related enzymes, PR-1a and β-actin in bacterial wilt pathogenesis of eggplant. A: Amplification of defense enzymes in resistant (R), susceptible (S) and highly Susceptible (HS) cultivars of eggplant seedlings upon pathogen inoculation. After Semi-quantitative RT-PCR was carried out, equal volume of 10 µl of total DNA was loaded in the gel. The data showed is a one representative example of three independent experiments.

GPOD defense gene plays several important roles in disease resistance expressed against a number of pathogens. Increases in GPOD activity are often associated with progressive incorporation of phenolic compounds within the cell wall during incompatible plant-microbe interactions or elicitor treatment. In tomato, GPOD is one of the enzymes believed to catalyse the last step in lignifications. The reinforcement of the plant cell wall by phenolics and lignin increases plant resistance to cell wall-degrading enzymes and toxins produced by pathogens, and acts as mechanical barrier to physical penetration of the cell. Increase in GPOD activity in ASM-treated plants may cause oxidative cross-linking of proteins to increase the resistance against bacterial pathogens. Other physiological processes induced as a defense response to pathogens in which GPOD is involved include the deposition of polyphenols and cross-linking of cell wall proteins. Induction of GPOD has been implicated in production of toxic radicals, such as O^{2-}, and H_2O_2. In plants, the increased production of both the superoxide radical and H_2O_2 is a common feature of defense responses to challenge by avirulent pathogens and elicitors [39]. The expression of G*POD* gene in treated plus pathogen-inoculated plants showed a tendency of induction immediately after the infection (0 hpi) with a slight increase of 1.1-fold, which was then significantly enhanced to 2.1-fold at 24 hpi over the pathogen-infected control alone. In addition, treatment alone resulted in a significant upregulation of G*POD* by an 8.5- and 12.3-fold increase at 4 h and 24 h, respectively, over the SDW-treated control. Similarly, in our present study the fold change in defense expression of GPOD activity was higher in resistant cultivar followed by susceptible and highly susceptible cultivars compared to their respective control.

Total phenol assay

Rapid increase in total phenolic content at 24 hpi was observed in R cultivar (Arka Shirish). In R cultivar the total phenolic content was 87.79 μg phenol g-1 in un-inoculated control, which was significantly increased to 236. 2 µg phenol g-1. The level of total phenolic content was 2.7 folds higher compared to control seedlings. The total phenol content in the S cultivar (MEBH-11) was 80.80 μg phenol g-1 in control and was increased to 171 μg phenol g^{-1} when inoculated with pathogen. On the contrary HS which did not exhibit much variation among inoculated and control plants (Figure 3). However, among fifteen cultivars, R cultivars exhibited the highest phenolic content followed by S and HS cultivars.

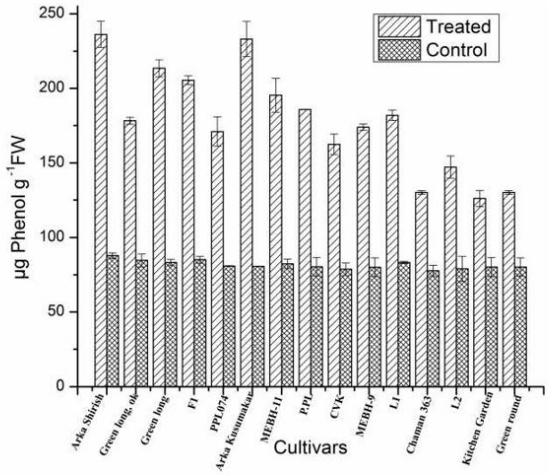

Figure 3: Effect of *R. solanacearum* infection on total phenolic content in different eggplant cultivars at 24 h with their controls. The values are means of three replicates ± standard deviation in significance of $P<0.05$. Bars indicate standard errors.

In our previous reports the activity of phenols was evaluated in the host pathogen interaction of *R. solanacearum* and tomato by Vanitha et al. [32], which revealed the accumulation of phenols with respect to R, S and HS cultivars. These antioxidant properties play an important role in quenching ROS by their Redox properties. Phenols and polyphenols are the non-enzymatic antioxidant ROS scavengers which are widely distributed in plant species, which activate defense mechanism. In present study, a significant increase in the levels of phenols and polyphenols was observed after 24 hpi in inoculated plant. Among the 15 cultivars the total phenol content was significantly enhanced upon pathogen inoculation, in which R cultivar (Arka Shirish) exhibited significant increase in phenols (3 folds) compared to control and least content was observed in MEBH 11 cultivar. The significant difference in phenols and polyphenols were described by Mandal et al. [23] in response of *F. oxysporum*-tomato, *Oidiumneo lycopersici*-tomato interaction in susceptible genotypes of tomato. Further, Bean plant response to Bean yellow mosaic virus and Salicylic acid interaction was evaluated with accumulation of total phenols scavenging activity [40]. These results support, that phenols and polyphenolic compounds play an important role in scavenging free radicals with respect to different cultivars of eggplant. Treatment of tomato seedlings with LAB resulted in high phenol accumulation in plant extracts. Seedlings treatment of LAB resulted in maximum

accumulation of phenolics (340 μg^{-1} mg^{-1} catechol) when compared to the control (120 μg^{-1} mg^{-1} catechol). The phenolics accumulation increased after challenge inoculation with *R. solanacearum* and reached the maximum level on 24 h. whereas, similar results were observed in the present study that in resistant cultivar of eggplant accumulation of phenolics was high when treated with pathogen [38].

The general trends of developmental regulation of total phenolics and GPOD in plants were a steady increase with germination and decrees pathogenesis. This is justifiable because higher phenolic synthesis was required for lignifications with growth and defense against pathogenesis. Meanwhile several reports explained GPOD play fundamental roles such as lignifications of cell wall, oxidation of hydroxy-cinnamyl alcohol into free radical intermediates act as potential biochemical marker. The developed novel biochemical and molecular marker play a significant role in screening resistant and susceptible cultivars against bacterial wilt by specific and sensitive increase in activity of GPOD and total phenols in eggplant.

Conclusion

Our study was an effort to analyze the activity of Guaiacol peroxidase in bacterial wilt pathogenesis of Brinjal. Out of the two cultivars selected, the cultivar- MEBH-9 showed signs of susceptibility to bacterial wilt after pathogen inoculation and the Green variety showed signs of resistance by increase in Guaiacol peroxidase enzyme activity. Simultaneously, there was a significant increase in total phenol in the resistant cultivars with respect to their controls. The developed assay can be used as a Biochemical marker for screening of cultivars against bacterial wilt in *Solanum melongena* and the developed molecular markers assist for specific, easy and rapid identification of *R. solanacearum* which overcomes the laborious and confusing conventional techniques.

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