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Potential biocontrol agents involved in induction of defense related enzymes in brinjal (*solanum melongena* l.) Against phomopsis blight caused by *phomopsis vexans*

ROHINI M., [1]NAVEEN J., [2]PUSHPALATHA H.G., [3*]HITHAMANI G., [4]HARIPRASAD P., AND [5]NIRANJANA S.R.[1]

[1]Department of Biotechnology, University of Mysore, Mysore-570 006, Karnataka, India

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ABSTRACT

Phomopsis blight, caused by Phomopsis vexans *(*P. vexans*) in brinjal plants is one of the main diseases in India. In order to manage this malady, we initially screened seed samples of 14 cultivars obtained from seed suppliers for the disease incidence. Based on the disease incidence and severity, the samples were categorized as resistant and susceptible cultivars of brinjal. It was established that MEBH-9' was a sensitive cultivar, while the Kolar local was resistant to the disease. Two ideal strains of Rhizosphere colonizing bacteria (RCB),* Pseudomonas putida *strain Has-1/c (HM229805), and Phylloplane Colonizing Bacteria (PCB),* Bacillus subtilis *strain Br/ph-33 (KJ867501), previously characterized as potential plant growth-promoting rhizobacteria, were tested for their potential biocontrol of this disease. Both susceptible and resistant cultivars' seedlings were treated with different combinations of PCB and RCB, PCB alone, RCB alone, and challenge inoculated with* P. vexans*. In order to ascertain whether there was signalling between defense enzyme activation and plant protection after PCB and RCB treatment, defense enzymes were quantified. Brinjal plants treated with PCB+RCB combination and challenge inoculated with* P. vexans *showed a significant increase in the activity of Phenylalanine ammonia-lyase (PAL), Peroxidase (POX), Lipoxygenase (LOX), Polyphenol oxidase (PPO), Catalase, Chitinase, β -1,3-glucanase, and Hydrogen peroxide (H_2O_2) content. The current study reveals that the defense enzymes and PR-proteins are gradually induced and accumulated, which enhance the resistance in brinjal plants against* P. vexans*-causing fruit rot disease.*

Keywords: Brinjal (*Solanum melongena* L.), Bio-control agents, Defense-related enzymes, Enzyme induction, *Phomopsis vexans*

INTRODUCTION

Vegetables are essential for human nutrition, providing a variety of nutrients and reduce the risk of long-term health issues. Solanaceous vegetables, such as tomatoes, egg plants, and chili peppers are protective foods since they are rich in phytonutrients, dietary fibers, minerals, and major vitamins. Eggplant is nutritious, rich in Vitamins A and B, economical, and has health benefits like supporting heart health, aiding digestion, preventing cancer, promoting bone health, preventing anemia, and improving brain function. Wild Solanum species are being used in the pharmaceutical sector (Quamruzzaman *et al.,* 2020). Eggplant, also known as brinjal, is one of the five most significant vegetables worldwide; the estimated total world production of eggplants worldwide in 2022 was 103,680,000 mt. China accounted for approximately 64.58 % of the world's production, with India producing 12,800,000, t., making it the second largest producer (FAO, 2022). Plants lose quality and yield as a result of pathogen infestations interfering with physiological and metabolic processes in the plants. Pathogens disrupt the normal physiological and metabolic functions as well as defense mechanisms in plants, leading to decreased yield and compromised quality. Eggplant is susceptible to various diseases, and one of the main issues affecting the growth and yield of brinjal production is Phomopsis blight, which is caused by *Phomopsis vexans* (*P. vexans*) having widespread occurrence (Bhanushree *et al.,* 2022). This fungal disease has a major negative influence on eggplant production, leading to lower-quality, considerably fewer fruits, which leads to a huge economic impact. At present, *P. vexans* is widely distributed in all major brinjal growing regions of the world. It causes damping-off of seedlings in nursery, leaf blight; fruit rot and stem blight at various stages of plant growth and development. *P. vexans* is both soil and seed-borne, persists on the seed surface and inside the seed, and is spread by rain splash (Mahadevakumar and Janardhana, 2015).

Corresponding author: pushpanarayana2012@gmail.com [2] Department of Biochemistry, CFTRI, Mysore-570 006, Karnataka, India, [3] Department of Botany, Government College for Women (Autonomous), Mandya-571401, Karnataka, India, [4] Fruit and Vegetable Technology, CFTRI, Mysore-570 006, Karnataka, India, [5] Centre for Rural Development and Technology, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110016

Quantification in disease prevalence across six agro-ecological regions and its severity demands accurate disease prediction and development of better management strategies (Mahadevakumar and Janardhana, 2016a and b).

The management of fungal diseases requires an integration of multiple strategies, such as exclusion (using certified seed), eradication (sanitizing farm equipment), and protection (chemical and biological control; Nolte *et al.,* 2020). The increasing use of fertilizers, pesticides, and fungicides for food production threatens human health and the environment. Alternative, environmentally friendly methods are needed to control pests and diseases. Even though a lot of plant extracts are quite effective in controlling *P. vexans*, these can be modified based on what plant material is readily available in large quantities (Reddy *et al.,* 2018). Biological management strategies have emerged as potential alternatives to chemical applications for crop disease prevention. Plant growth-promoting rhizobacteria (PGPR) are group of microorganisms with stimulatory and protective effects on plants. Biological control agents like *Bacillus subtilis* and *Pseudomonas putida* are essential for suppressing crop diseases and pests and also have a growth-promoting effect on the plant. Bacteria are ubiquitous and colonize leaf surfaces, and studies on potential phylloplane colonizing bacteria (PCB) in improving plant growth, productivity (Tyagi and Durgapal, 2019; Manju and Prabakaran, 2020) and suppressing foliar phytopathogens (de Almeida Halfeld-Vieira *et al.,* 2015) are sporadically reported.

Plants generate reactive oxygen species (ROS) under biotic and abiotic stresses, which can cause oxidative damage to proteins, lipids, and photosynthetic pigments, as well as inactivate photosynthetic enzymes (Singla *et al.,* 2019). Plants have evolved complex defense mechanisms to protect themselves against pathogen attacks, which can be constitutive or induced and fail when infected by virulent pathogens (Pieterse and van Loon, 1999). Major induced changes in host plants during pathogen infection include the induction of reactive oxygen species, activation of defense mechanisms comprising of enzymatic and non-enzymatic antioxidative components, secondary metabolites, pathogenesis-related protein expression, production of phytoalexins, modification of cell wall composition, production of melatonin, accumulation of carotenoids, and altered activity of polyamines, therefore, disease development is restricted by the changing concentration of biochemical components in host plants (Kaur *et al.,* 2022). Some rhizosphere colonizing bacteria trigger defense mechanisms by activating some defense related enzymes in plants, making susceptible cultivars resistant to pathogen infection (Saeed *et al.,* 2021). The induced defense mechanism includes various non-enzymatic components comprising phenolic compounds, flavonoids, lignins and enzymes for phenol metabolism like phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO) and antioxidant enzymes like Catalase (CAT), Peroxidases (POX). Defense enzymes play a crucial role in plant defense against pathogens, where oxidative enzymes like POX and PPO catalyze the formation of lignin and other oxidative phenols, which contribute to the formation of defense barriers for reinforcing cell structure. Enzymes like PAL are involved in phytoalexins or phenolic compound biosynthesis (Senthilraja *et al.,* 2013). Lipoxygenases (LOXs) catalyze the peroxidation of polyunsaturated fatty acids and lipids, resulting in the production of oxylipins and biologically active compounds (Yang *et al.,* 2012). These enzymes enhance defense responses by directly inhibiting pathogens and accumulating phytoalexins (Lin and Ishii, 2009).

Pathogenesis-related (PR) proteins like chitinases (PR-3 family and β-1, 3-glucanase (PR-2 family) degrade the fungal cell wall, causing the lysis of the fungal cell (Kauffmann *et al.,* 1987). The phenolic compounds released during degradation act as elicitors, eliciting various defense mechanisms in plants. The induction of defense-related enzymes makes plants resistant to pathogen invasion. This has been correlated with defense mechanisms against pathogen invasion in plants like tomato (Bashan *et al.,* 1985) cucumber (Rasmussen, 1991) and tobacco (Binutu and Cordell, 2000). In addition to self-defense of plants, useful microbes and plant growth-promoting rhizobacteria also activate the defense mechanism *via* two different pathways, systemic acquired resistance (SAR) and induce systematic resistance (ISR). The ISR may be strengthened through beneficial microorganisms, whereas SAR implies an altered gene

expression at molecular levels and is related to PR proteins (Kaur *et al.,* 2022). Reports suggest that certain strains of PGPR can stimulate defense mechanisms in plants, leading to systemic disease protection, ISR (Van Loon, 2007). This can protect even susceptible plants by enhancing their own defense mechanisms upon different applications of PGPR. The PGPR has been reported to induce defense enzymes against various diseases, such as *Fusarium oxysporum* in tomato (Ramamoorthy *et al.,*2002a), *Pythium aphanidermatum* in tomato and hot pepper (Ramamoorthy *et al.,* 2002b), *Rhizoctonia solani* in tomato (Solanki *et al.,*2012), and *Colletotrichum capsici* in pepper (Ramanujam, *et al.,* 2012). Studies on PGPR-mediated ISR have shown the role of defense enzymes such as PO, PPO, PAL, β-1,3-glucanases, and chitinases in induced systemic resistance (Jetiyanon, 2007). In this present study, we describe how brinjal cultivars are categorized as resistant or susceptible against bacterial blight disease caused by *P. vexans*, under greenhouse environments. The second goal of this research was to decipher how different combinations of treatment with a well-characterized PCB, *Bacillus subtilis* (strain Br/ph-33; KJ867501), and RCB, *Pseudomonas putida* (strain Has-1/c; HM229805), along with challenge inoculated with *P. vexans*, induce the activity of defense-related enzymes in resistant and susceptible cultivars.

MATERIALS AND METHODS

Seed Samples

A total of 14 different commonly growing seeds of brinjal cultivars were purchased from the local seed agencies of Mysore-570 006, Karnataka, India. Seeds were surface sterilized by using 1 % sodium hypochlorite (NaOCl) for 30 sec and then washed thoroughly with sterile distilled water to remove surface treated chemicals, blot dried and were grown under greenhouse conditions (day 25° C/night 20° C with 80 % relative humidity (RH) in an earthen pot (20 cm diameter) containing soil: sand: farmyard manure (2:1:1) (Table 1).

Phomopsis vexans

In our previous study, a total of 23 suspected *P.vexans* isolates from different parts of infected brinjal leaves, fruits and seeds were subjected to various biochemical physiological and molecular characterizations to deduce the virulent and avirulent pathogen. Among the 23 isolates of *P. vexans,* Pv1 (Accession No. KF994965) recorded the highest disease incidence (97 %) and severity (88.6 %) with high activities of Cellulase (CL), catalase (CAT) and ascorbate peroxidase (APX) enzymes (Rohini *et al.,* 2023). Fungal inoculum for greenhouse studies was prepared by growing it on PDA for 14 days under near ultraviolet (NUV) radiation (365 nm). Towards the end of the incubation period, conidial ooze from pycnidia was collected by flooding the plate with 10 ml of sterile distilled water. The concentration of conidia was adjusted to 1×10^8 conidia/ml using haemocytometer.

Table 1: List of seed samples of brinjal subjected to screening to assess their resistance and susceptibility to *P. Vexans*

Sl.No	Cultivar
1	F1 Green Long
2	CVK
3	Ravaira Hybrid
4	MEBH-9
5	Chaman-363 F1 Hybrid
6	Pragathi
7	SHB-211 F1 Hybrid
8	Brinjal F1 Hybrid
9	Kolar Local Variety
10	F1 Abhishek
11	Mohini
12	Padma
13	Ramya
14	Apsara

Phylloplane Colonizing Bacteria (PCB) and Rhizosphere Colonizing Bacteria (RCB)

In our present experiment we have used a PCB strain Br/Ph-33, which was previously characterized by biochemical and molecular methods, which upon sequencing using 16s rRNA, analysis proved the strain belongs to *Bacillus subtilis* (strain Br/ph-33 (KJ867501), which showed 49.5 % inhibition against the pathogen. Foliar application of the isolate showed significant difference in root and shoot length compared to control. The disease incidence was suppresses by 45 % and disease severity was recorded to be 2.24 (Rohini *et al.,* 2016). This study has used another well characterized *Pseudomonas putida* strain Has-1/c (HM229805), which showed 44.4 % inhibition against the pathogen. Sequencing also confirms the strain belonging to *Pseudomonas putida.*

This strain showed plant growth promotion with increase in root; shoot length, fresh and dry weight of brinjal seedlings. The disease incidence was 30.8 % and disease severity of 2.53 was recorded upon RCB inoculation (Rohini *et al.,* 2016).

Screening of Brinjal Cultivars for Resistance/ Susceptibility against *Phomopsis vexans*

In order to evaluate the resistance/ susceptibility of different brinjal cultivars against selected highly virulent isolate of *P. vexans* 'Pv1', the greenhouse experiments were conducted by growing the healthy seedlings raised from surface sterilized seeds in pots (one seedling/pot) containing sterilized soil: sand: farm yard manure (2:1:1 @ v/v/v).Thirty-day-old seedlings were spray-inoculated with conidial suspension of P. *vexans* (1× 10^8 conidia ml^{-1}) till runoff. Development of leaf blight disease was regularly monitored based on development of leaf blight symptoms. After 15 days post inoculation, the disease severity was assessed by using 0–9 scale (Mayee and Datar, 1986). The experiment was laid out in Randomized Complete Block Design (RCBD) manner with three replications and repeated three times. The Phomopsis leaf blight disease incidence and severity was calculated using the standard formula (Rohini *et al.,* 2023).

Induction of Defense Mechanisms by Defense-Related Enzymes

To study the activation of defense related enzymes, two cultivars resistant cultivar (Kolar local variety) and Susceptible ('MEBH–9') were selected. The temporal pattern study of these enzymes was carried out using the susceptible seedlings. Fifteen-days-old seedlings were challenge inoculated with the conidial suspension of *P. vexans* as described above. The PCB isolate Br/ph-11 and RCB isolate Has-1/c effective bacteria was used to assess their efficiency to induce defense mechanisms by defense-related enzymes in brinjal seedlings against *P. vexans* under greenhouse conditions.

The following treatments were included in the greenhouse experiment: (1) seedlings raised from untreated seeds; (2) seedlings raised from untreated seeds and challenge inoculated with *P. vexans*; (3) seedlings raised from RCB treated seeds; (4) seedlings raised from untreated seeds and spray inoculated with PCB; (5) seedlings raised from RCB treated seeds and spray inoculated with PCB; (6) seedlings raised from RCB treated seeds and challenge inoculated with *P. vexans*; (7) seedlings raised from untreated seeds, spray inoculated with PCB and challenge inoculated with *P. vexans* and (8) seedlings raised from RCB treated seeds, spray inoculated with PCB and challenge inoculated with *P. vexans*. Ten pots for each treatment were maintained and arranged in a completely randomized block design in a greenhouse with three replications and the experiment was repeated three times.

Sample Collection for the Analysis of Induction of Defense Mechanisms

Seedlings raised from each treatment were carefully uprooted without causing any damage to root tissues at different time intervals *viz*., 0, 6, 12, 24, 36, 48, 72, 96, 120 and 144 h after challenge inoculation with *P. vexans*. Six plants were randomly sampled from the different treatment separately for each biochemical analysis and were maintained separately. The seedlings were washed in running tap water and homogenized with liquid nitrogen in a pre-chilled pestle and mortar in sodium borate buffer for PAL, sodium phosphate buffer for PO and PPO, sodium citrate buffer for Chitinase and sodium acetate buffer for β-1,3 glucanase activity at 4° C. The homogenized tissues were stored at −80° C and used for biochemical analysis.

Phenylalanine Ammonia Lyase (PAL; EC. 4.3.1.5) Activity

PAL activity was assayed by following the method of Lisker *et al*. (1983). One gram of brinjal seedlings was homogenized in 3 ml of ice cold 0.1 M sodium borate buffer (pH 7.0) containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinylpyrrolidone (PVP). The resulting extract was filtered through cheese cloth and the filtrate was centrifuged at 10000 rpm for 15 min at 4° C. The supernatant was used as the enzyme source. The PAL activity was determined spectrophotometrically as measurement of rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. The reaction mixture contained 1m of enzyme extract incubated with 0.5 ml of 0.1 M borate buffer (pH 8.8) and 0.5 ml of 50 mM L-phenylalanine in the same buffer for 30 min at 30° C. The amount of trans-cinnamic acid

produced was calculated using its extinction coefficient of 9630 M^{-1} cm^{-1}. The enzyme activity was expressed as nmol trans-cinnamic acid min^{-1} mg^{-1} of protein.

Peroxidase (POX; EC. 1.11.1.7) Activity

POX activity was assayed as described by Hammerschmidt et al. (1982). One gram of brinjal seedlings was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4° C. The homogenate was centrifuged at 10000 rpm for 15 min at 4° C. The supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1 % H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2° C). The changes in absorbance at 420 nm were recorded at 30 sec intervals for 3 min. The boiled enzyme preparation served as the blank and the enzyme activity was expressed as changes in the absorbance at 420 nm/min^{-1} mg^{-1} of protein.

Polyphenol Oxidase (PPO; EC. 1.14.18.1) Activity

PPO activity was determined as described by Mayer et al. (1965). One gram of brinjal seedlings was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4° C. The homogenate was centrifuged at 10000 rpm for 15 min at 4° C. The supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M catechol was added and the enzyme activity was expressed as changes in absorbance at 495 nm min^{-1} mg^{-1} of protein.

Lipoxygenase (LOX; EC. 1.13.11) Activity

LOX activity was determined as described by Borthakur et al. (1987). One gram of brinjal seedlings was homogenized in 1 ml of 200 mM sodium phosphate buffer (pH 6.5) at 4° C. The homogenate was centrifuged at 12,000 rpm for 20 min at 4° C. The supernatant served as the enzyme source. The activity was determined spectrophotometrically by monitoring the appearance of conjugated diene hydroperoxide, absorbing at 234 nm. The reaction mixture contained 2.7 ml of 0.2 M sodium phosphate buffer (pH 6.5), 0.3 ml of 10 mM linoleic acid in Tween-20 and 50 ml of the enzyme extract. The enzyme activity was expressed as a change in the absorbance at 234 nm min^{-1} mg^{-1} of protein.

Catalase (CAT; EC 1.11.1.6) Activity

CAT activity was determined as described by Chance and Meahly (1955). One gram of brinjal seedlings was homogenized in 1 ml of 50 mM phosphate buffer (pH 7.0) at 4° C. The homogenate was centrifuged at 10000 rpm for 20 min at 4° C. The supernatant served as the enzyme source. The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2 and 50 µl of the enzyme extract. The enzyme activity was determined spectrophotometrically by decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2. The activity was expressed as a change in the absorbance at 240 nm min^{-1} mg^{-1} of protein.

Chitinase (EC 3.2.1.14) Activity

Chitinase activity was carried out as per the method described by Boller and Mauch (1988) by using colloidal chitin as substrate. Colloidal chitin was prepared according to Berger and Reynold (1958) from crab shell chitin (Sigma, USA). One gram of brinjal seedlings was extracted with 2 ml of 0.1 M sodium citrate buffer (pH 5.0) at 4° C. The homogenate was centrifuged at 10000 rpm for 15 min at 4° C and the clear supernatant was used as the enzyme source. The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml of colloidal chitin (10 mg). After 2 h incubation at 37° C, the reaction was stopped by centrifugation at 8000 g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3 per cent (w/v) desalted snail gut enzyme (Helicase) for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 µl 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled in an ice-water bath. Finally, the mixture was incubated with 2 ml of dimethyl amino benzaldehyde (DMAB) for 20 min at 37° C. Immediately thereafter, the absorbance was measured at 585 nm. N-acetylglucosamine (GlcNAc) was used as a standard. The enzyme activity was expressed as nmoles GlcNAc min^{-1} mg^{-1} of protein.

β-1,3-glucanase (EC 3.2.1.6) Activity

β-1, 3-glucanase activity was assayed by the laminarin-dinitrosalicylic acid method (Pan *et al.,* 1991). One gram of brinjal seedlings was extracted with 2 ml of 0.05 M sodium acetate buffer (pH 5.0) at 4° C. The homogenate was centrifuged at 10000 rpm for 20 min at 4° C. The clear supernatant was used as the enzyme source. The reaction mixture consisted of 62.5 µl of 4 % laminarin (Sigma, USA) and 62.5 µl of the enzyme extract. The reaction was carried out at 40° C for 10 min and stopped by adding 375 µl of dinitrosalicylic acid and heated for 5 min on boiling water, vortexed and its absorbance was measured at 500 nm. The enzyme activity was expressed as µg glucose released min^{-1} mg^{-1} of protein.

Hydrogen Peroxide (H_2O_2) Content

The quantitative production of H_2O_2 was determined according to the method of Patterson *et al.* (1984) with slight modifications as previously described by Aroca *et al.* (2003). Five hundred milligram of brinjal leaf sample was homogenized in a cold mortar with 5 ml (5 %; w/v) TCA containing 0.1 g of activated charcoal and 1 % (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 10000 rpm for 10 min. The supernatant was filtered used as the enzyme source. The reaction mixture consisted of 1.2 ml of 100 mM potassium phosphate buffer (pH 8.4) and 0.6 ml of the colorimetric reagent and 130 µl of the enzyme extract. The colorimetric reagent was freshly prepared by mixing 0.6 mM potassium titanium oxalate and 0.6 mM 4-2 (2-pyridy-lazo) resorcinol (disodium salt) 1:1 (v/v). The samples were incubated at 45° C for 1 h and the absorbance was recorded at 508 nm. The blanks were made by replacing leaf extract by 5 % TCA. The accumulation of H_2O_2 was expressed as nmol H_2O_2 min^{-1} mg^{-1} of protein.

The H_2O_2 accumulation in the brinjal leaves collected from the plants inoculated with combination application of PCB and RCB was visualized using light microscopy. Briefly, the leaves collected at different time intervals after inoculation with *P. vexans* were immersed in a solution containing 1.0 mg DAB (3´-3´-diaminobenzidine) ml^{-1}. The DAB was freshly dissolved in Milli-Q grade water adjusted initially to pH 3.0 with 1 N HCl and heated to 50° C and followed by the addition of 1 N NaOH (pH 4.0). After 8 h treatment, the leaves were decolorized by incubation in 1.5 g l^{-1} TCA in 3:1 (v/v) mixture of ethanol + chloroform for 48 h with at least three changes of the bleaching solution and examined by light microscopy. To detect papilla (callose) formation, the leaf pieces were soaked with phosphate buffer (0.1 M KH_2PO_4/ K_2HPO_4, pH 9) for 24 h, and the leaf pieces with infection sites were placed on glass slides in phosphate buffer (Barreto *et al.,* 2007).

Subsequently, the samples were stained with 0.1 g l^{-1} buffered solution of aniline blue for 2 h (Borden and Higgins, 2002). The leaf pieces were mounted on a glass slide, cleared by drop washing with concentrated HCl and covered with a glass cover slip in glycerol (Mlícková *et al.,* 2004). The experiments were repeated three times with three replications.

STATISTICAL ANALYSIS

The laboratory and greenhouse experimental data were statistically analyzed separately and subjected to arcsine transformation and analysis of variance (ANOVA) using SPSS, ver. 17 (SPSS Inc., Chicago, IL). The significant differences between the treatment means were compared using Highest Significant Difference (HSD) as obtained by Tukey's test at $p \leq 0.05$ level.

RESULTS AND DISCUSSION

Evaluation of Resistance/ Susceptibility of Brinjal Cultivars against *Phomopsis vexans*

In the present study, a total of 14 different brinjal cultivars were screened against a highly virulent *P. vexans* strain Pv1 for their resistance/ susceptibility. Among 14 cultivars screened, the cultivar 'Kolar local variety' was found more resistant to *P. vexans* infection which recorded the disease incidence and severity of 30.6 and 47.0 %, respectively. The cultivar 'MEBH–9' showed significantly ($p \leq 0.05$) highest disease incidence and severity of 99.0 and 97.0 %, respectively. This susceptible cultivar (MEBH–9) was used for further studies (Fig. 1). Our findings align with those of Islam and Meah (2011), who conducted screenings of brinjal seeds across various regions of Bangladesh to determine the prevalence of *P. vexans*. They documented the highest disease incidence at 7.5%.

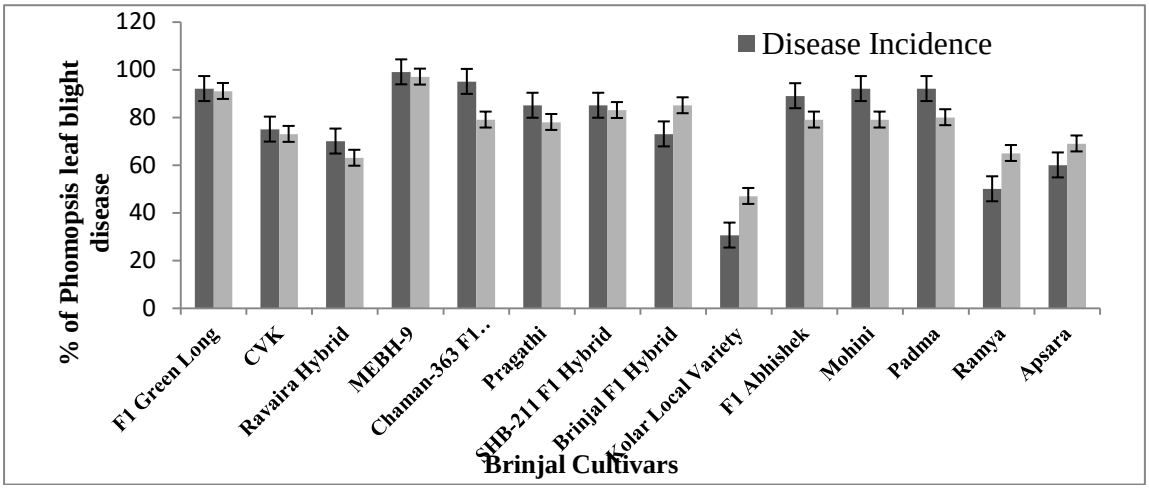

Fig. 1: Evaluation of Resistance/Susceptibility of different brinjal cultivars against *Phomopsis vexans*. The date expressed is the mean of three separate experiments with three replicates

In Karnataka, Mahadevakumar and Janardhan (2015) reported a wide range of Phomopsis blight from 7.7 to 30.5 % with fruit rot ranging from 21.0 to 64.6 %. Jayaramaiah *et al.,* (2013) reported that *P. vexans* caused leaf blight to the extent of 5 to 23 % and fruit rot to the extent of 30 to 60%. Mahadevakumar *et al.,* (2017) reported leaf blight incidence (8 to 25 %) and fruit rot incidence (15 to 62 %) caused by *P. vexans*. However the leaf incidence was relatively low varying from 8.81 to 23.85 % among 25 varieties of brinjal screened. Recently Prasad *et al.* (2022) reported a disease severity of brinjal plants against *P. vexans* in 32 genotypes which varied from 0 to 13.75 %.

Induction of Defense related enzymes

Mild and virulent strains of bacteria, such as *Pseudomona*s, *Bacillus, Trichoderma* and *Aspergillus* can mediate**,** impart and induce resistance in a plant system. This method has also been shown to be environmentally benign, economical, and beneficial to both the plant and the soil and water bodies (Ahamed *et al.,* 2022). Biological control agents like *Bacillus subtilis* are essential for suppressing crop diseases and pests. *Pseudomonas* is considered one of the most promising groups as potential biofertilizers due to their numerous plant growth-promoting traits with *P. putida* being one of the best studied *Pseudomonas* PGPR strains (Costa-Gutierrez *et al.,* 2020a; 2020b). Plant growth-promoting rhizobacteria can induce systemic resistance to various plant diseases. Combining bio-control agents can provide better disease protection than individual treatments due to different defense enzyme mechanisms. Singh and Jha (2016) concluded that PGPR strains alone or in combination can provide effective, economical, and practical plant protection against multiple pathogens, high-lighting the potential of PGPR in promoting plant health. Researchers have recognized the antagonistic activities of *Bacillus* and *Pseudomonas*, with several *Bacillus* species causing ISR and protecting plants from pathogen attacks (Kashyap *et al.,* 2021). Some of the *Pseudomonas putida* strains exhibit outstanding traits like phytohormone synthesis, nutrient solubilization, adaptation to stress, and excellent root colonization ability among various PGPR species (Garcia-Gutierrez *et al.,* 2022). The present study was carried out to study various defense related enzymes using two very well characterized PCB (strain Br/Ph-33) and RCB (Has-1/c (HM229805) used in combination for treatment of Brinjal plants infected with *P.vexans*. In order to follow the temporal pattern for the defense related gene like PAL, POX, PPO, LOX, Catalase, Chitinase, β-1,3-glucanase and H_2O_2, the seedlings of the susceptible brinjal cultivar MEBH–9' was selected to treat with different combinations of PCB and RCB were challenge inoculated with *P. vexans*. The different formulation of *B. subtilis* (strain Br/ph-33 and *P. putida* strain Has-1/c (PCB+RCB) strains varied in their efficacy on defense enzyme activity in Brinjal plants inoculated with *P.vexans*.

PAL: The plants were induced to synthesize PAL in the brinjal seedlings raised from seeds treated with PCB isolate Br/ph-11 and RCB isolate Has-1/c alone or in combination.

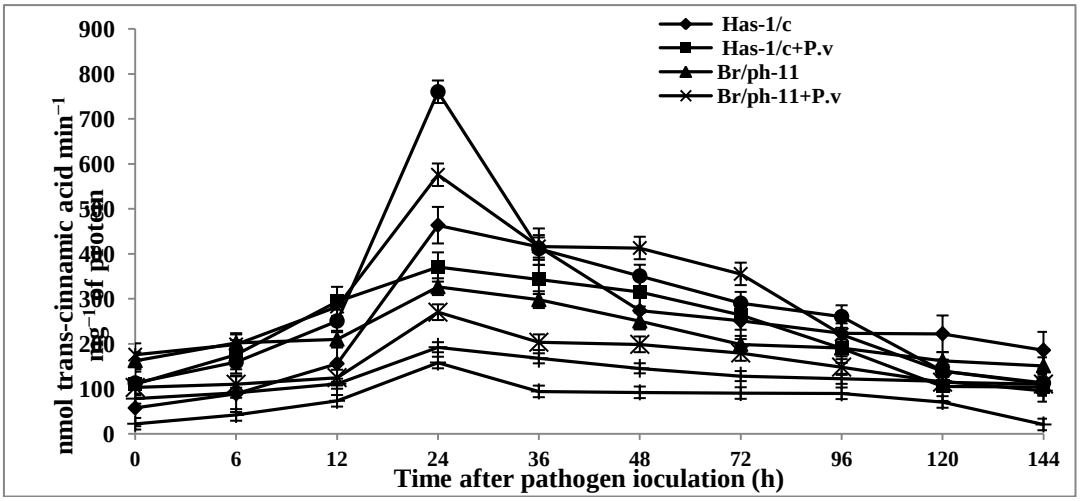

Fig. 2A: Temporal pattern of PAL activity in brinjal seedlings treated with PCB and RCB alone or in combination against *P. vexans* under greenhouse conditions. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

However, compared to control, there was an additional increase in synthesis in the PCB and RCB alone or in combination pretreated plants challenge inoculated with *P. vexans*. The enzymes' temporal pattern analysis revealed that the enzyme activity peaked 24 hours after the pathogen was inoculated, and that it thereafter steadily decreased over the course of the trial (Fig. 2A).To study the induction pattern of this enzyme, after 24 hours post-infection, seedlings of resistant and susceptible cultivars were analyzed. In the susceptible cultivar, when seedlings treated with PCB+RCB and challenge inoculated with *P. vexans,* the enzyme activity increased from 120 nmol trans-cinnamic acid min^{-1} mg^{-1} and 150 nmol trans-cinnamic acid min^{-1} mg^{-1} of protein respectively to 730 nmol trans-cinnamic acid min^{-1} mg^{-1} of protein. A comparable pattern was seen in the resistant cultivar (Kolar local variety), where seedlings treated with PCB+RCB after pathogen inoculation displayed an enzyme activity of 880 nmol trans-cinnamic acid min^{-1} mg^{-1} of protein, in contrast to seedlings treated with *P. vexans* and its control (118 and 740 nmol trans-cinnamic acid min^{-1} mg^{-1} of protein, respectively (Fig. 2B).

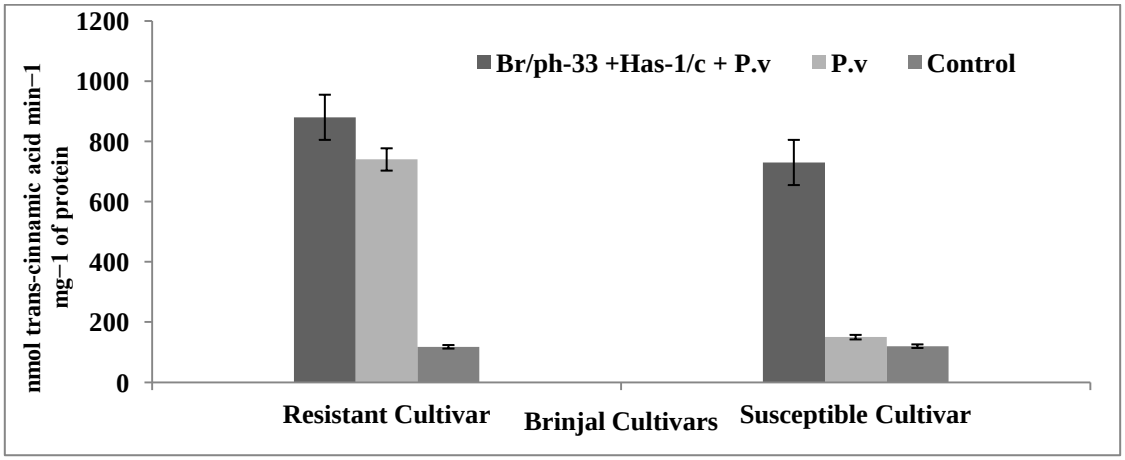

Fig. 2B: Induction of PAL in PCB+RCB combination-treated resistant and susceptible brinjal seedlings at 12hpi, followed by *P. vexans* challenge inoculation. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

POX: In the seedlings grown to study the temporal pattern of the POX enzyme, the combination treatment caused maximum activity of the enzyme 12 hours after inoculation with *P. vexans* compared to the control, and the activity then gradually decreased (Fig. 3A). Following *P. vexans* inoculation, POX activity considerably increased in the PCB+RCB treated brinjal plants

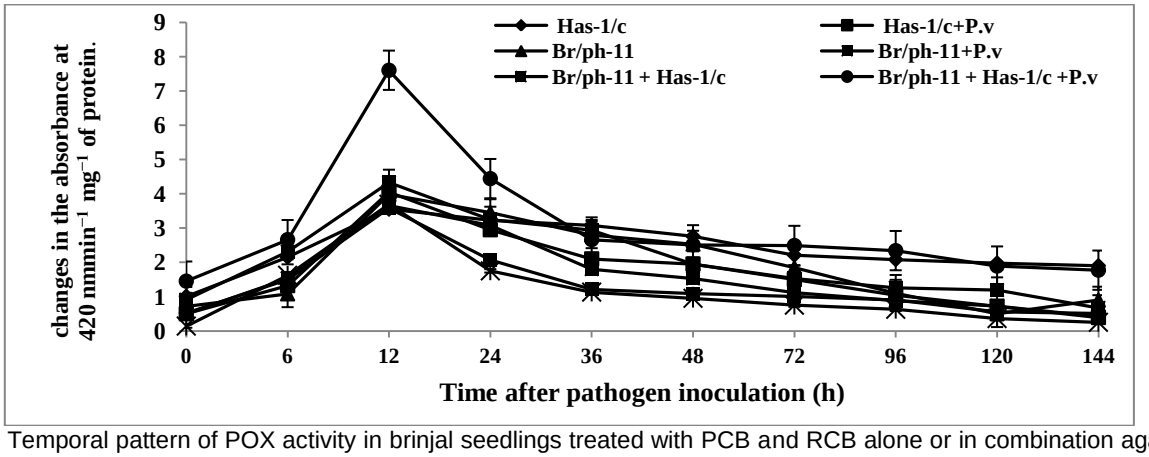

Fig. 3A: Temporal pattern of POX activity in brinjal seedlings treated with PCB and RCB alone or in combination against *P. vexans* under greenhouse conditions. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

compared to the untreated inoculated and non-inoculated control, indicating further research into the induction pattern of this enzyme in resistant and susceptible cultivars. In resistant cultivar, the non-inoculated control, the activity increased to 7.93 nmmin^{-1} mg^{-1} of protein upon *P. vexans* inoculation. However, the enzyme's maximum activity was 9.85 nmmin^{-1} mg^{-1} of protein in PCB+RCB treated seedlings. When compared to untreated and non-inoculated seedlings, of both resistant and susceptible cultivar, which displayed 3.8 and 3.6 nmmin^{-1} mg^{-1} of protein, respectively, POX demonstrated a higher level of activity (7.85 nm min^{-1} mg^{-1} of protein) in seedlings with PCB+RCB combination in the susceptible cultivar (Fig. 3B).

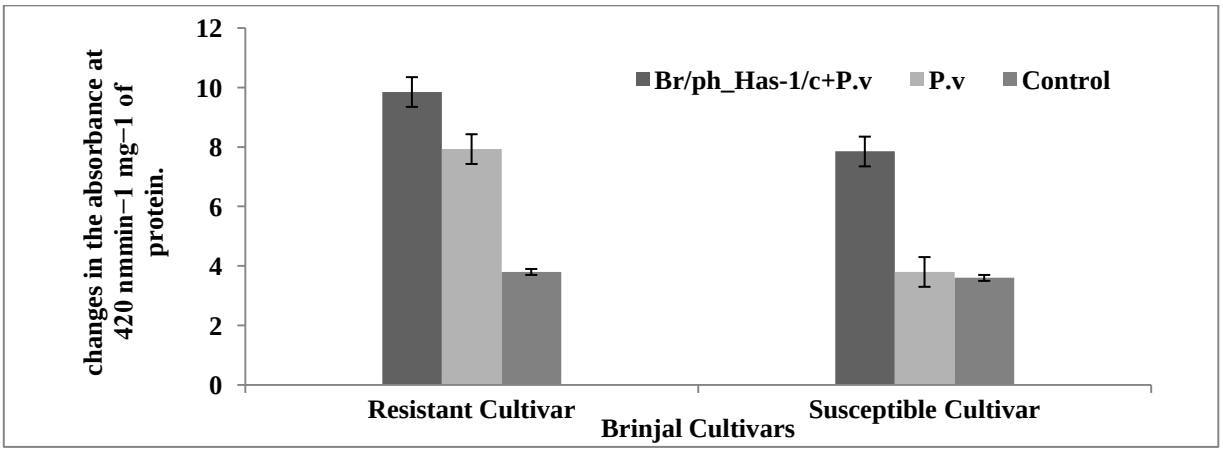

Fig. 3B: Induction of POX in PCB+RCB combination-treated resistant and susceptible brinjal seedlings at 24 hpi, followed by *P. vexans* challenge inoculation. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

PPO: When compared to other treatments and the control, the combination treatment containing PCB and RCB showed the highest activity in the examination of the PPO enzyme's temporal pattern, 24 hours after the *P. vexans* challenge. Subsequently, the activity gradually decreased (Fig. 4A). Compared to plants treated or untreated at 24 hours post-harvest, plants treated with combinations of PCB+RCB strains exhibited a greater induction of PPO. Lesser PPO activity was seen in susceptible cultivar treated with *P.vexans* and non-inoculated seedlings (1.02 nm min^{-1} mg^{-1} of protein and 0.8 nm min^{-1} mg^{-1} of protein respectively). However, PPO activity rose in the seedlings treated with PCB+RCB (5.01 nm min−1 mg−1 of protein) (Fig.4B)

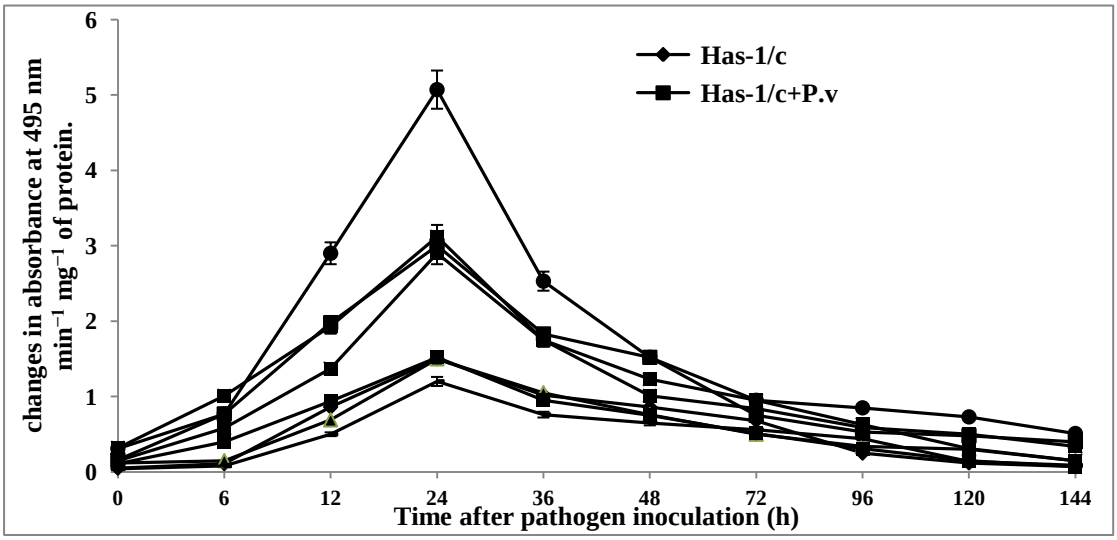

Fig. 4A: Temporal pattern study of PPO activity in brinjal seedlings treated with PCB and RCB alone or in combination against *P. vexans* under greenhouse conditions. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

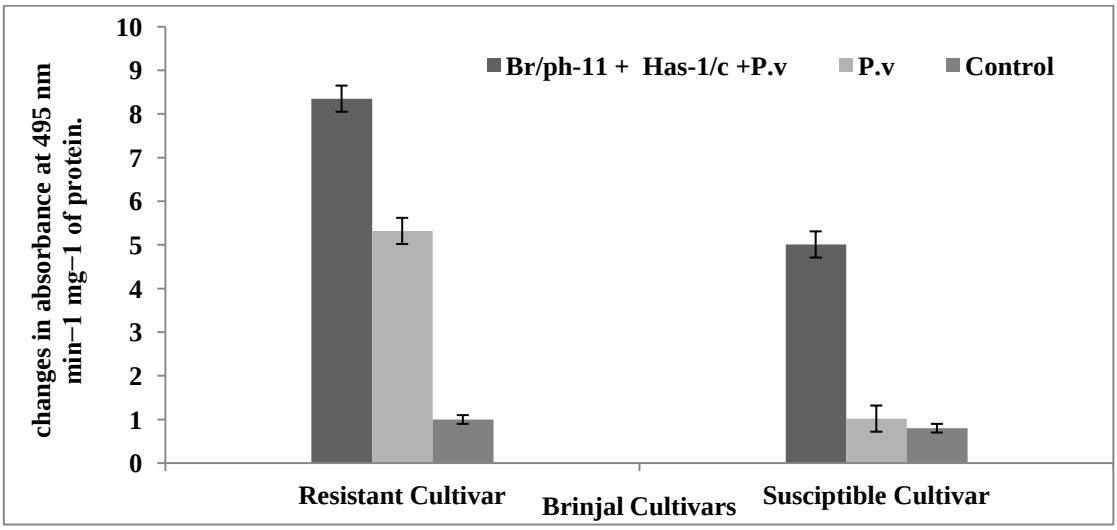

Fig. 4B: Induction of PPO in PCB+RCB combination-treated resistant and susceptible brinjal seedlings at 24 hpi, followed by *P. vexans* challenge inoculation. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

LOX: With respect to LOX, activity was observed 12 hours after pathogen inoculation in all treatments compared to the control, with the combination of PCB and RCB exhibiting the highest activity (Fig. 5A). A test of LOX activity in response to different treatments clearly showed that brinjal seedlings treated with PCB+RCB strains and challenge inoculation with the *P. vexans* pathogen at 12 hours after pathogen inoculation had higher activity in both susceptible and resistant cultivars. In PCB+RCB treated resistant and susceptible seedlings upon challenge inoculation showed LOX activity of 12.32 nm min^{-1} mg^{-1} of protein and 9 nm min^{-1} mg^{-1} of protein respectively which was higher when compared to their respective untreated inoculated and non-treated seedlings (Fig. 5B).

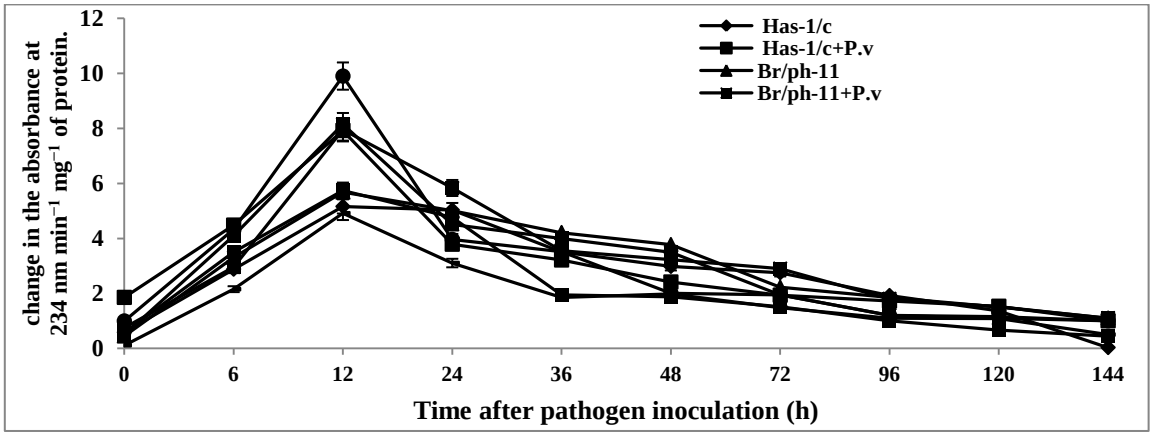

Fig. 5A: Temporal pattern study of LOX activity in brinjal seedlings treated with PCB and RCB alone or in combination against *P. vexans* under greenhouse conditions. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

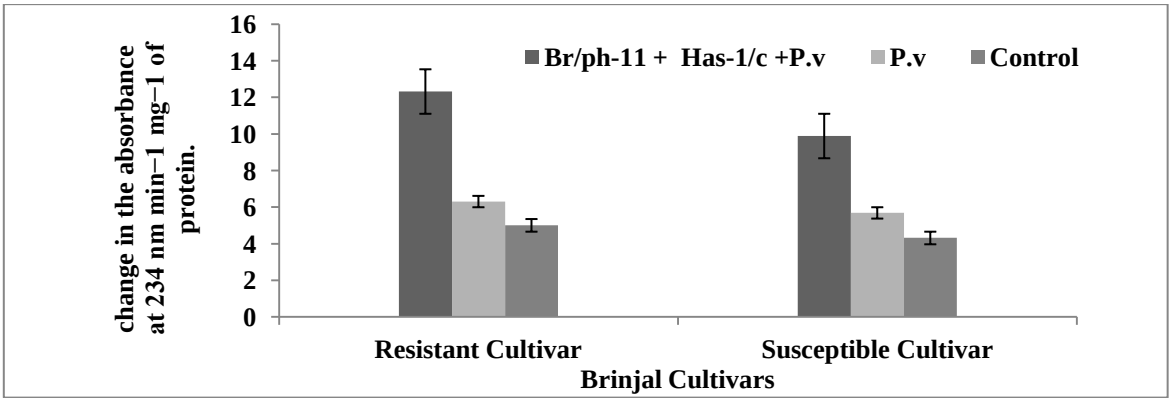

Fig. 5B: Induction of LOX in PCB+RCB combination-treated resistant and susceptible brinjal seedlings at 12 hpi, followed by *P. vexans* challenge inoculation. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

CAT: The temporal pattern changes in catalase activity showed a maximum induction in all the treatments at 24 hours after pathogen inoculation (Fig. 6A). In both resistant and susceptible cultivars, the untreated inoculated and non-inoculated plants had significantly reduced levels of catalase activity. Compared to other treatments, the application of PCB+RCB increased the expression of catalase in Brinjal seedlings. In resistant seedlings treated with PCB+RCB and challenge inoculated with *P. vexans*, the activity was observed to be 6.9 nm min^{-1} mg^{-1} of protein compared to its control at 24 hours after pathogen inoculation (Fig. 6B).

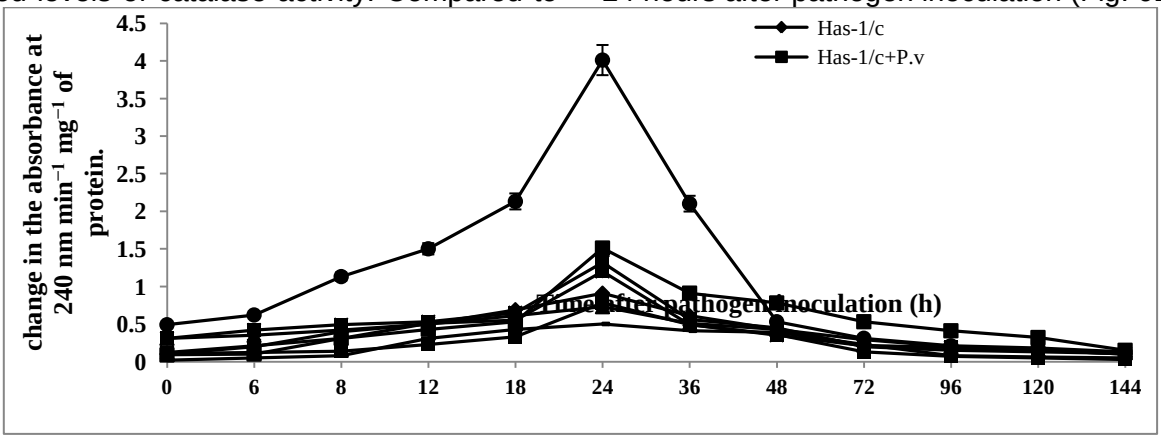

Fig. 6A: Temporal pattern study of CAT activity in brinjal seedlings treated with PCB and RCB alone or in combination against *P. vexans* under greenhouse conditions. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

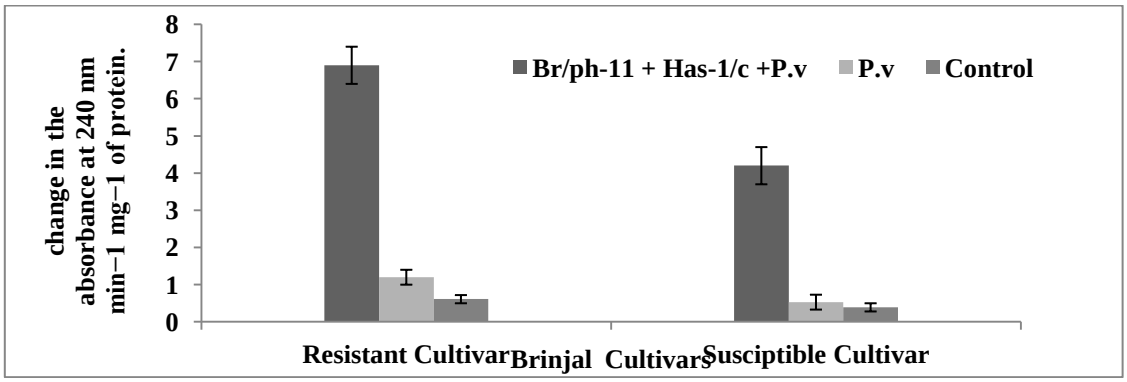

Fig. 6B: Induction of CAT in PCB+RCB combination-treated resistant and susceptible brinjal seedlings at 24 hpi, followed by *P. vexans* challenge inoculation. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

Chitinase: The efficacy of combination of PCB+RCB in increase of chitinase enzyme activity was seen in susceptible brinjal cultivar when compared to resistant cultivar. PCB+RCB treated seedlings upon challenge inoculated with *P.vexans* showed an increase in enzyme activity at 24 hours after pathogen inoculation *i.e* 9.68 nmoles GlcNAc min^{-1} mg^{-1} of protein, where the untreated inoculated and non-inoculated seedlings showed 5.9 nmoles GlcNAc min^{-1} mg^{-1} of protein and 5.3 nmoles GlcNAc min^{-1} mg^{-1} of protein, respectively. When challenging the resistant cultivar with *P. vexans*, PCB+RCB treated seedlings showed an increase in enzyme activity to 12.3 nmoles GlcNAc min^{-1} mg^{-1} of protein, while untreated inoculated seedlings exhibited 10.19 and non-treated seedlings showed 5.9 nmoles GlcNAc min^{-1} mg^{-1} of protein (Fig. 7A and 7B).

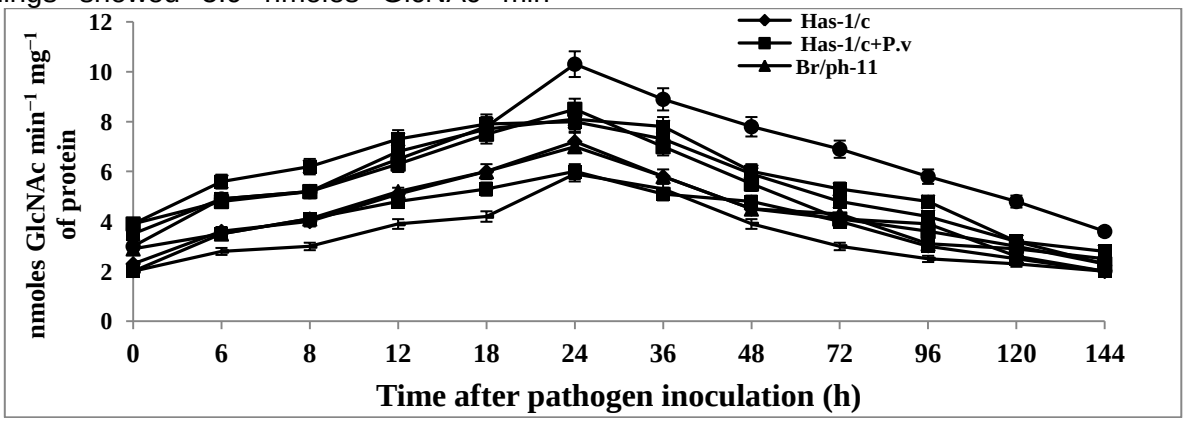

Fig. 7A: Temporal pattern study of Chitinase activity in brinjal seedlings treated with PCB and RCB alone or in combination against *P. vexans* under greenhouse conditions. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

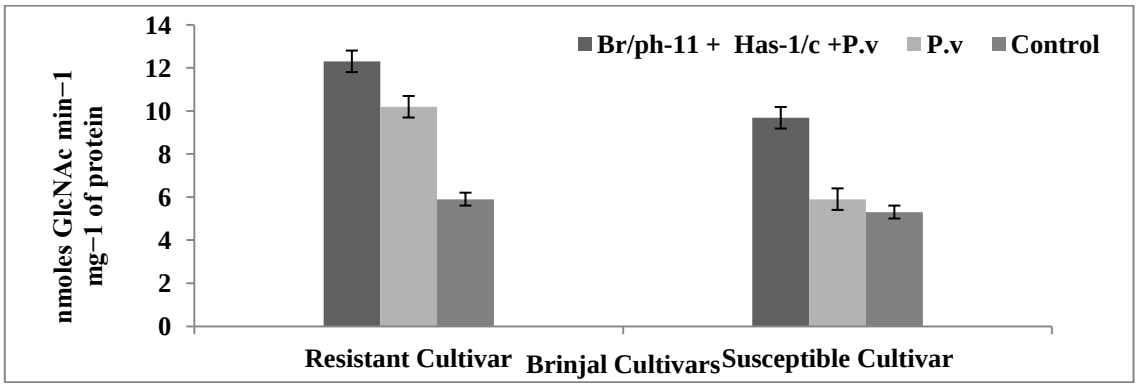

Fig. 7B: Induction of Chitinase in PCB+RCB combination- treated resistant and susceptible Brinjal seedlings at 24 hpi, followed by *P. vexans* challenge inoculation. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

β-1,3-glucanase : Naturally, β-1,3 glucanase gene expression levels are relatively low, but when a plant–pathogen interaction or elicitors are used, high levels of β-1,3 glucanase can be detected; enzyme accumulation occurs rapidly and consequently hydrolytic activity increases (Ali *et al.,* 2021). In our temporal pattern studies for the β-1,3-glucanase enzyme, PCB and RCB combination showed the highest level of activity at 24 hours after the susceptible seedlings were inoculated with the pathogen (Fig. 8A). Pathogen-treated and non-treated susceptible seedlings displayed an activity of 23 min^{-1} mg^{-1} of protein and 13 min^{-1} mg^{-1} of protein at 24 hours post-infection. In contrast, the β-1,3-glucanase enzyme activity caused by PCB and RCB treated susceptible seedlings was measured to be 52 min^{-1} mg^{-1} of protein in susceptible cultivar. The enzyme activity in non-treated resistant seedlings was 18 min^{-1} mg^{-1} of protein, which upon pathogen inoculation was 60 min^{-1} mg^{-1} of protein, but in PCB+RCB treated challenge inoculated seedlings, an highest activity of 79 min^{-1} mg^{-1} of protein was observed (Fig. 8B). In the present study we found that the expression β-1,3-glucanase , chitinase and H_2O_2 production was high in the seedlings treated with two PGPR and challenge inoculated with the fungal pathogen, compared to their respective controls. Our findings are correlated with many reports which showed that after fungal infection, *β*-1, 3-glucanases expressed in coordination with chitinases as cited from different crops like bean, pea, tomato, maize, tobacco, soybean, wheat, barley and potato (Sels *et al.,* 2008).

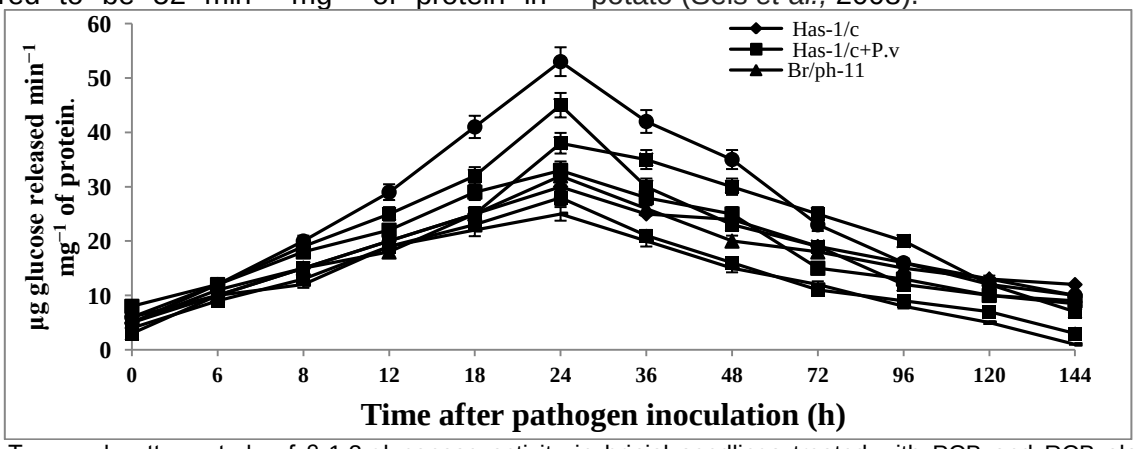

Fig. 8A: Temporal pattern study of β-1,3-glucanase activity in brinjal seedlings treated with PCB and RCB alone or in combination against *P. vexans* under greenhouse conditions. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05.

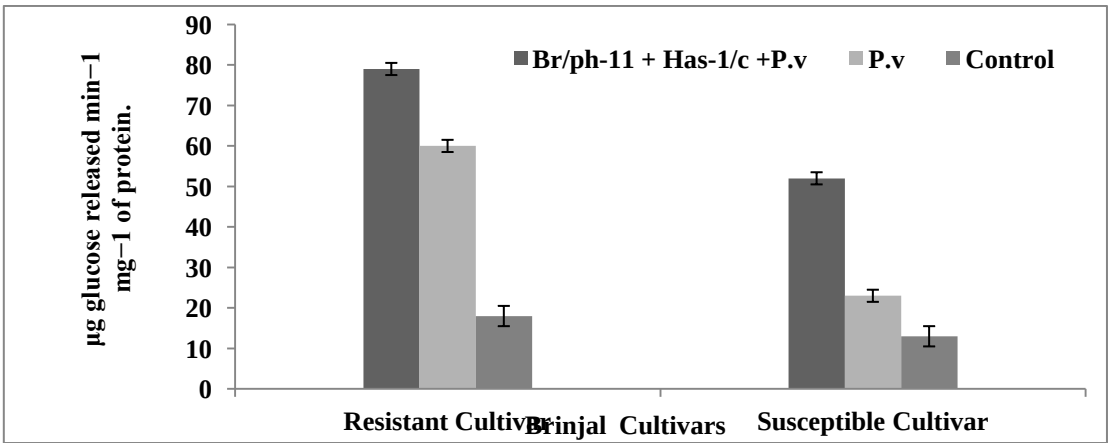

Fig. 8B: Induction of β-1,3-glucanase in PCB+RCB combination-treated resistant and susceptible Brinjal seedlings at 24 hpi, followed by *P. vexans* challenge inoculation. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

H_2O_2 : To overcome pathogen infection, plants deploy a highly efficient innate immune system, which often uses hydrogen peroxide (H_2O_2), a versatile reactive oxygen species, to activate downstream defense responses. Increased catalase activity is thought to be an adaptive

characteristic that could aid in overcoming tissue metabolic damage by lowering harmful levels of H_2O_2 In addition, H_2O_2 is a critical component of stress response regulation in crop plants such as rice (Sohag *et al.,* 2020), wheat (Habib *et al.,* 2020), maize (Terzi *et al.,* 2014), soybean (Guler *et al.,* 2016), cucumber (Sun *et al.,* 2016). It's known that upon contact with PGPR, plant H_2O_2 levels often increase and H_2O_2 accumulation can be primed for enhanced resistance against pathogens. Studies on the trend of H_2O_2 accumulation patterns in susceptible cultivar at various time intervals with varying combinations of treatments revealed that it peaked at 24 hours post-inoculation stage(Fig. 9A). The resistant cultivar showed an accumulation of 0.20 nmol H_2O_2 min^{-1} mg^{-1} of protein in seedlings treated with PCB +RCB where its non-treated seedlings showed 0.1 nmol H_2O_2 min^{-1} mg^{-1} of protein. The H_2O_2 accumulation was 0.19 nmol H_2O_2 min^{-1} mg^{-1} of protein in seedlings of untreated inoculated. The change in the accumulation pattern was significant in the susceptible seedlings treated with PCB+RCB which were 0.15 nmol H_2O_2 min^{-1} mg^{-1} of protein when compared to its non-inoculated control which showed 0.1 nmol H_2O_2 min^{-1} mg^{-1} of protein (Fig. 9B). With these previous reports, our findings on H_2O_2 accumulation increasing pattern in susceptible seedlings when treated with combination of two PGPR strains are correlated.

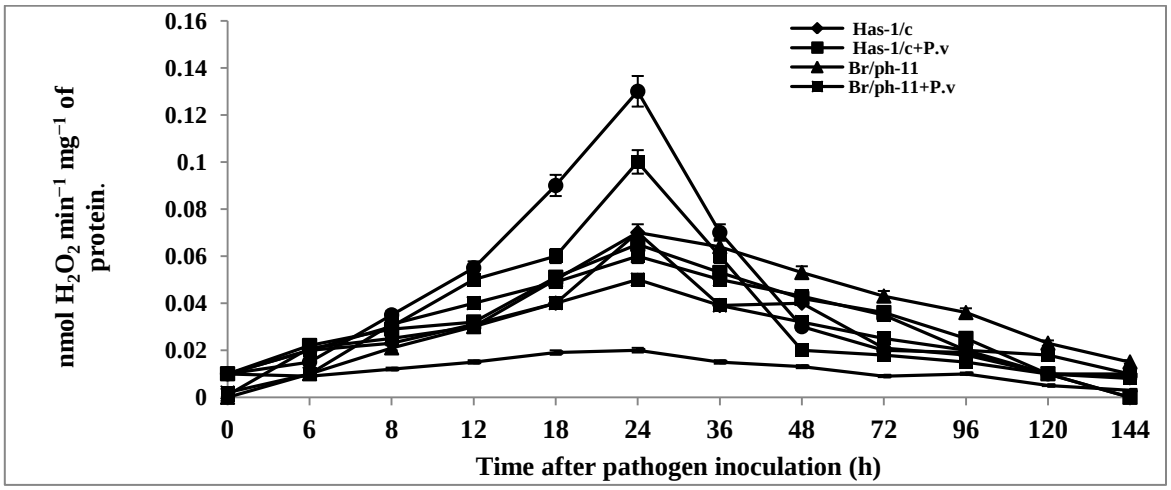

Fig. 9A: Temporal pattern study of H_2O_2 activity in brinjal seedlings treated with PCB and RCB alone or in combination against *P. vexans* under greenhouse conditions. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

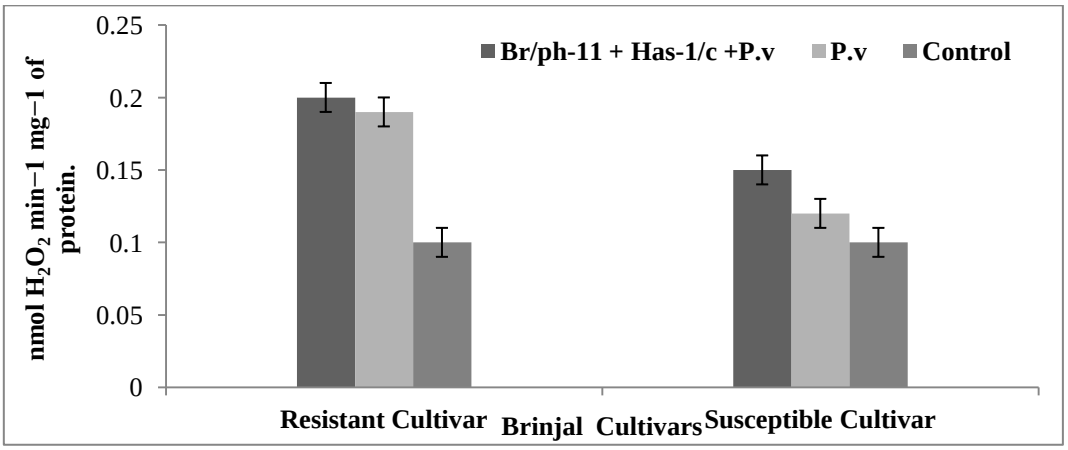

Fig. 9B: Induction of H_2O_2 in PCB+RCB combination-treated resistant and susceptible brinjal seedlings at 24 hpi, followed by *P. vexans* challenge inoculation. The data are expressed as the mean of three separate experiments with three replicates each. All treatments are significantly different from each other at P≤ 0.05

Our results are consistent with the work of other researchers who have established that integrated plant resistance (ISR) is a viable approach in controlling plant diseases that can effectively shield a plant from the respective diseases. In our investigation, we discovered

that combination treatment of *P. putida* strain Has-1/c and *B. subtilis* strain Br/ph-33) demonstrated the effectiveness of generating distinct defense-related enzymes in resistant (kolar local variety) and susceptible (MEBH-9) seedlings. The enzyme activities of PAL, POX, PPO, LOX, Chitinase, β-1,3 glucanases, and H_2O_2 were examined temporally in susceptible seedlings. The results indicated that the seedlings treated with *P. putida* strain Has-1/c and *B. subtilis* strain Br/ph-33, challenge inoculated with *P. vexans*, had varying times for these enzyme activities compared to their untreated inoculates and non-inoculated seedlings. we observed that PAL, PPO, Chitinase, β-1,3 glucanases, and H_2O_2 accumulation increased at 24 hours after pathogen inoculation, but POX and LOX activity peaked at 12 hours after pathogen inoculation.

Our results also correlate with previous investigations which revealed that different PGPR strains protect the plants from various pathogens by activating plant defense genes encoding chitinase, β-1,3 glucanase, PAL, CAT, APX, POX and other enzymes, many of which act as primary ROS scavengers (Singh *et al.,* 2020). In our findings, we established that in susceptible seedlings treated with combination of two different PGPR's and challenge inoculated with the pathogen *P.vexans*, showed an increase in the level of defense related enzymes when compared to their controls and also when compared to the resistant seedlings with the same treatment. Ghazy and Nahrawy (2021), revealed similar finding where antioxidant enzyme activity (CAT, POX and PPO) significantly improved in plants exposed to inoculation treatments with *B. subtilis* and *P. koreensis* either alone or in combination, which in turn reflected in the reduced disease incidence of *Cephalosporium maydis* in maize plant. Our results are in consonance with the other findings where plants inoculated with *P. oryzae* in the absence of bio-control agents exhibited reduced PO, SOD, PPO, and PAL activity, demonstrating the connection between oxidative damage and the ROS scavenging system. However, in response to *P. oryzae* infection, the antagonistic *Bacillus* sp. markedly increased the activity of antioxidant enzymes in rice shoots and roots by about a 2- to 5-fold. Kashyap *et al.,* (2021), found that treatment of chili plants with *Bacillus subtilis* KA9 and *Pseudomonas fluorescens* PDS1 resulted in increased defense enzyme activities like PAL, POX, PPO and resulted in the induction of resistance in chili against *Ralstonia solanacearum*. Similarly, the interaction between pathogens and host plants causes some changes in the activity of certain enzymes, including phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, lipoxygenase, superoxide dismutase, and β-1,3-glucanase (Manzar *et al.,* 2021a,b). Sentilraja *et al.* (2013) recorded the assay of various defense related enzyme activity from different combination treatments of PGPR (*B. bassiana* and *P. fluorescens* strains (Pf1 and TDK1) and *Beauveria bassiana* (B2) which clearly showed higher activity in groundnut plants challenge inoculated with leaf miner larvae or collar rot pathogen. Similar results were observed in majority of the cases like, in rice shoot lysates, rhizobacterial treatment increased the activity of the PAL enzyme (Bhattacharyya *et al.,* 2020). Van Loon (1997) reported that the Chitinases and β-1,3 glucanases have been associated with plant resistance against fungal pathogens. Similar results have also been observed in case of PR proteins such as chitinases and β-1,3-glucanase. The increased enzyme activity observed in the PCB and RCB treated seedlings facilitated efficient detoxification of H_2O_2 generated following *P. vexans* inoculation, highlighting their potential in combating pathogen-induced stress in plants.

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