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Induction of Systemic Resistance in *Persea bombycina* Against *Pestalotiopsis disseminata* Using Bioinoculants

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ABSTRACT

Among eight different morphotypes (S1–S8) of *Persea bombycina,* locally known as som plant, screened for resistance against *Pestalotiopsis disseminata* causing grey blight disease, S5morphotype was found to be highly susceptible under field conditions. Immunodetection of *P. disseminata* in leaf tissue using Polyclonal antibody raised against the pathogen has been demonstrated following various immunological formats such as immunodiffusion, dot immunobinding assay, plate trapped antigen (PTA) coated enzyme linked immunosorbent assay (ELISA) and indirect immunofluorescence. Immunogold localization of grey blight pathogen (*P. disseminata*) in som leaf tissue using transmission electron microscopy has been demonstrated for the first time for this host-pathogen system .Strategies for induction of immunity in som plants (S5 morphotype) against *P. disseminata* using bioinoculants such as PGPF (*Trichoderma asperellum*), AMF (*Rhizophagus fasciculatus*) and PGPR (*Bacillus pumilus*) alone or in combinations have been developed. Plant growth promotion and reduction in disease severity were evident following application of bioinoculants. Significant increase in defense enzymes such as chitinase (CHT), β-1,3 glucanase (GLU), phenylalanine ammonia-lyase (PAL) and peroxidase (POX) were observed in both roots and leaves following the application of bioinoculants. Cellular localization of chitinase and glucanase in leaf and root tissue following induced immunity against grey blight pathogen using PAbs of chitinase and glucanase have been demonstrated by indirect immunofluorescence and immunogold labelling. It is clearly evident that the applications of bioinoculants greatly improved the health status of som plants (S5) and also induced systemic resistance in the plant against grey blight pathogen.

Keywords: *Persea bombycina*, *Pestalotiopsis disseminata*, Grey blight, Induced resistance, Bioinoculants

INTRODUCTION

Muga silk, popularly known as the “golden silk”, is one of the most precious silk fibers available on earth due to its uniqueness in the fibers and rarity in presence, as this silkworm is present only in the North-Eastern region of India. Although muga culture has a rich tradition and heritage, it is increasingly being threatened for its very survival due to the rampant and irrational exploitations (Tikader *et al*., 2011). Muga silk is produced by the silkworm *Antheraea assamensis* Helfer, a polyphagous insect feeding on a wide range of plants *viz*., som (*Persea bombycina*) and soalu (*Litsea monopetala*) being the primary host plants, and dighloti (*Litsea alicifolia*) and mejankori (*Litsea citrata*) as the secondary host plants. Although, efforts have been made to domesticate this silkworm by rearing them under captivity, not much success could be obtained, and hence, it is still left in the wilderness of the North eastern India which has distinct tropical humid climatic conditions with evergreen and deciduous forests. In order to provide a better shelter for this silkworm, efforts have been made to cultivate the host plants in the border regions of the forest (Thangavelu *et al*., 2005).

Som (*Persea bombycina*) belongs to the family Lauraceae, a medium sized evergreen tree with spreading branches, bark and foliage usually aromatic; alternate leaves grow abundantly in its natural habitat in Assam particularly Brahmaputra valley up to an elevation of about 500 meters. Besides, its distribution also extends to Khasi and Jayantia Hills in India, along the Lower Himalaya and as far as to the west of Nepal (Rahman *et al*., 2012). Muga culture has been confined to the north eastern states of India and to a small extent to the Coochbehar district of West Bengal. Due to evergreen nature of this food plant, muga silkworm can be reared on it throughout the year. The plants become suitable for rearing of muga silkworm after 3-5 years of growth and can be used until 20-25years (Singh and Sen, 2001). Som plant is vulnerable to many foliar diseases that affect the normal growth of the plant, quantity and quality of leaves and ultimately cocoon yield production and an estimated annual leaf yield loss of about 20-30% has been reported due to major fungal foliar diseases such as Leaf spot, Red rust, Leaf blight and Grey blight (Das *et al*., 2003). Grey blight caused by *Pestalotiopsis disseminata* has been reported as one of the major diseases of som plant, causing 13.8-41.6% leaf yield loss (Das and Benchamin, 2000).The disease is so severe that it leads to shortage of quality leaves for rearing of

muga silkworm finally causing severe economic loss to farmers. A knowledge of how the plants respond to infection, special biochemical and molecular responses would go a long way in attempts to induce resistance. Several studies along this line have been reported previously. Biochemical changes in *Persea bombycina* following infection with *Colletotrichum gloeosporioides* were demonstrated by Chakraborty *et.al*. (2016a).Pathogen (*C. gloeosporioides*) causing leaf blight disease in muga host plant triggered the production of resorcinol, catechol, chlorogenic acid, ferulic acid and salicylic acid. Characterization of bacteria from the rhizosphere of *Persea bombycina* with multiple growth promoting traits have been reported (Rabba *et al*., 2014).Activation of defense response in som plant against *C. gloeosporioides* (leaf blight pathogen) following application of bioinoculants have also been discussed (Chakraborty *et.al.*, 2016b).

Arbuscular Mycorrhizal Fungi (AMF) have a 90% symbiotic association with higher plants, which are being commercially exploited as bioinoculants to improve plant growth. Perspectives of arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria (PGPR) mediated plant disease management have been illustrated (Chakraborty and Chakraborty, 2012). Chakraborty (2019a) has elucidated molecular detection of AM fungi and their role in symbiosis and crop protection. Harnessing beneficial microbial resources, analysis of their diversity and development of bioformulations for crop improvement has been critically evaluated (Chakraborty, 2019b).

In the present study, attempts have been made to develop immunoassays for early detection of *Pestalotiopsis disseminata* in leaf tissues of *Persea bombycina* using PAb of pathogen; to develop strategies using bioinoculants such as PGPF (*Trichoderma asperellum*), AMF (*Rhizophagus fasciculatus*) and PGPR (*Bacillus pumilus*) for induction of systemic resistance in som plants against foliar fungal pathogen; to evaluate accumulation of defence enzymes triggered in leaf and root tissues of som plant following application of bioinoculants against *P. disseminata;* and cellular localization of chitinase and glucanase in leaf and root tissues following induced resistance against grey blight pathogen using PAbs of chitinase and glucanase by indirect immunofluorescence and immunogold labelling.

MATERIALS AND METHODS

Plant materials

Eight different morphotypes (viz. S1, S2, S3, S4, S5, S6, S7 and S8) of *Persea bombycina* were collected from Central Muga Eri Research and Training Institute, Jorhat, Assam. These were grown in earthenware pots (12" dia), maintained and kept in glass house. One year old saplings of all eight morphotypes were also transferred to experimental field of University of North Bengal where all suitable management practices were taken into consideration for proper growth of the plants.

Fungal Culture

The fungal pathogen isolated from naturally grey blight infected leaves of som plants was grown in PDA slant and after completion of Kochs' postulate, mycelial growth and sporulation were observed. The BLAST query of the 18S rDNA sequence of the pathogen confirmed its identity as *Pestalotiopsis disseminata* and have been deposited in NCBI, GenBank database (accession no. KT697994)

Artificial inoculation and Disease Assessment

Detached leaf inoculation

A detached leaf inoculation technique as described by Dickens and Cook (1989) was followed for artificial inoculation of leaves. Fresh, young, fully expanded som leaves detached from plants were placed in trays lined with moist blotting paper. Wounds were made on adaxial surface of each leaf with 26 G½ needle and inoculated with 20µl droplets of spore suspension (1.2×10^6 conidia ml^{-1}) of the fungus (prepared from 14 days old culture in PDA). Spore suspension was placed (2-4 drops per leaf) on the adaxial surface of each leaf with a hypodermic syringe on the wounds. In control sets drops of sterile distilled water were placed on the wounded leaves. Each tray was covered with a glass lid and sealed with petroleum jelly in order to minimize drying of the drops during incubation. Percent drops that resulted in lesion production were calculated after 48, 72 and 96 hours of incubation as described by Chakraborty and Saha (1994). Observations were made based on 50 inoculated leaves for each treatment in average of three separate trials.

Whole Plant inoculation

Whole plant inoculation was carried out essentially as described by Lakshmi *et al.* (2011) with minor modifications. The fungus was grown in PDA for 14 days at 30°±2°C and spore suspension was prepared (1.2×10^6 conidia ml^{-1}). Tween-20 was added @ 2ml l^{-1} to facilitate adhering of the spores to leaf surface. Two year old plants were spray-inoculated with an atomizer @ 100ml per plant so as to wet both ventral and dorsal surfaces. The plants were immediately covered with polythene bags in order to maintain high relative humidity and kept overnight. Next day, the polythene bags were removed and transferred to glass house benches and maintained at 30°±2°C. The disease severity on plant leaves was recorded using a 0-5

rating scale, where 0 = no infection; 1 = up to 5%; 2 = 6-10%; 3 = 11-20%; 4 = 21-50% and 5 = > 50% leaf area affected by the disease. Based on this numerical rating, a Percent Disease Index (PDI) was calculated using the formula: PDI = [total numerical ratings/(number of leaves examined × max rating scale)]× 100. Results were always computed as the mean of observations of 25 well-established and branched som plants in average of three separate experiments.

Bio-inoculants

Selection

The PGPF strain (*Trichoderma asperellum*-. NAIMCC-F-01963 NCBI Acc no HQ265418) and PGPR strain (*Bacillus pumilus*- NAIMCC-B01487 NCBI Acc No. JQ765579) were selected from culture collection of Immuno-Phytopathology laboratory which have been deposited at National Agriculturally Important Microbial Culture Collection (NAIMCC). The dominant AM Fungus (*Rhizophagus fasciculatus*) collected from the rhizosphere of som plant was used in this study after mass multiplication in maize plant.

Mass multiplication

PGPF*:* The selected strain of *Trichoderma asperellum* was initially grown on PDA. The sporulated culture was further mass multiplied in wheat bran medium supplemented with carboxymethyl cellulose and pH of the medium was maintained at 7 with Calcium carbonate. The spore suspension of *T. asperellum* was added to wheat bran medium and kept at room temperature till sporulation occurred 15 days after inoculation. This mass multiplied medium was then added in the rhizosphere region of the plants @ 200 g/pot.

AMF: Dominant spores of *R. fasciculatus* were washed with distilled water and inoculated to roots of 7-10 days old maize seedlings which were grown in Petri plates. After inoculation they were transferred to black plastic pots (12" diameter) filled with autoclaved soil. After 45 days the presence of AM spores was confirmed. Filter paper was cut into 5 mm dia circles and about 5-6 spores were transferred to the filter paper circles. The paper was then adhered to the roots of som saplings grown in pots with the help of tweezers. Besides, AMF spores colonized with maize roots were harvested and chopped for field application. Chopped roots colonized with AMF spores along with soil were applied to the rhizosphere of som plants.

PGPR*:* The selected strain of *B. pumilus* was grown in nutrient broth for 48 h and then centrifuged at 15000 rpm for 15 min. The pellet obtained was re-suspended in sterile distilled water. The optical density of this suspension was measured using a UV-VIS Spectrophotometer at 600 nm, to obtain a final density of $3x10^6$cfu/ml. A few drops of Tween20 were added to the bacterial aqueous suspension and applied as foliar spray as well as soil drench @ 200ml/pot.

For talc-based formulation, 10 g of carboxy methyl cellulose sodium salt (Himedia) was mixed with one kg of talcum powder and pH was adjusted to 7.0 by adding calcium carbonate. It was then sterilized twice for 30 min each. To 1 kg of sterilized talcum powder 400 ml of bacterial inoculum (aqueous suspension) was added and mixed well under sterile condition. The talc mix was dried under shade to bring moisture to less than 20%. The formulation was packed in milky white colour polythene bags to eliminate UV exposure, sealed and stored at room temperature for future use. The talcum based formulation was applied at the rate of 100 g per pot.

Application

In case of pot treatment, AMF spores were added to the roots of som sapling using filter paper disc. After 25 days of AMF treatment, *T. asperellum* in wheat bran medium (PGPF) was added to the soil. Two weeks after application of PGPF, foliar spray as well as soil drench application of PGPR were done thrice at 5 days interval. In case of joint treatment with all three bioinoculants (AMF, PGPF and PGPR), they were added to the pots sequentially but in case of dual treatments (AMF+PGPF; AMF+PGPR and PGPF+PGPR) applications were done accordingly. For field inoculation, chopped maize roots colonized with dominant spores of *R. fasciculatus* were applied in the root rhizosphere following transplantation in the field from nursery- grown 7 month old plants. One month following application of AMF, root colonization status was examined. Then *T. asperellum* mass multiplied in wheat bran was applied in soil. Two weeks after application of *T. asperellum*, further soil application of talc based formulation as well as foliar spray of *B. pumilus* were done.

Evaluation of plant growth promotion

Plant growth promotion was studied in terms of increase in height, number of leaves and number of branches after different treatments. Observations on growth parameters were recorded after 30 and 60 days of application in potted condition and after four months in case of field condition.

Extraction and assay of defense enzymes

For enzyme assays, freshly harvested leaves collected from Som plants following treatments, were crushed separately in 0.1M Sodium phosphate buffer pH 6.8 for peroxidase (POX), 0.05M Sodium acetate buffer pH 5.0 for glucanase (GLU) and chitinase (CHT), 0.1 M Sodium borate buffer pH 8.8 for phenylalanine ammonia lyase (PAL),

centrifuged and the supernatant collected was used as the enzyme source. POX and PAL were assayed following the method of Chakraborty *et al.* (1993). Method of Boller and Mauch (1988) was followed for Chitinase (CHT) activity, whereas β-1,3 Glucanase (GLU) activity was measured according to the method described by Pan *et al.* (1991).

Preparation of antigens, production of antisera and purification of IgG

Isolated clean spores of *R. fasciculatus* were separately sonicated with 0.1% normal saline under the frequency range of 70-75 Mhz as an impulse. The supernatant was used as an antigen source of *R. fasciculatus*. Mycelial antigen of *P. disseminata* was prepared following the method of Chakraborty *et.al*. (1995). Four New Zealand white male rabbits each of approximately 2 kg of body weight were immunized separately with fungal antigens prepared from *R. fasciculatus* and *P. disseminata* as well as chitinase and glucanase following the protocol described by Chakraborty and Saha (1994). Before immunization with fungal antigens, chitinase and glucanase, normal sera were collected from each rabbit. For developing antisera, intramuscular injections of 1ml of antigen mixed with 1ml of Freund's complete adjuvant (Genei) were given to each rabbit 7 days after pre immunization bleeding. The doses were repeated at 7 days intervals for the consecutive week, followed by Freund's incomplete adjuvant (Genei) at 7 days intervals up to 12-14 consecutive weeks as required. Marginal ear vein puncture was done for blood collection three days after the first six injections and then every fourth injection. Collected blood samples were incubated at 37^0 C for 1 h for clotting. After clotting, the clot was loosened with a sterile needle. Finally, the antisera were clarified by centrifugation, distributed in 1 ml vials, and stored at -20^0C until required. IgG was purified by DEAE Sephadex column chromatography following the protocol as described by Clausen (1988).

Serological assays

Agar gel double diffusion tests were performed following the method of Ouchterlony (1967). Mycelial antigen of *P. disseminata* as well as healthy and artificially inoculated (with *P. disseminata*) som leaf antigens were loaded on nitrocellulose membrane filter using Bio-Dot apparatus (Bio-Rad) and using PAb of pathogen Dot immunobinding assay (DIBA) was performed as outlined by Lange *et.al.* (1989). Plate trapped antigen coated enzyme linked immunosorbent assay (PTA-ELISA) was performed as described by Chakraborty *et.al*. (1995).

Indirect immunofluorescence

Purified IgG raised against *P. disseminata* (fungal pathogen), *R. fasciculatus* (AMF), chitinase and glucanase were used separately and labeled with goat antisera specific to rabbit globulins conjugated with fluorescein isothiocyanate (FITC) for indirect immunofluorescence study of conidia of *P. disseminata*, AM fungal spore associated with rhizosphere as well as grey blight infected leaf tissue, as well as cellular location of glucanase and chitinase in leaf and root tissue of som plant after induction of immunity by bioinoculants following the protocol described by Chakraborty (2021). Observations were made using a Biomed microscope (Leitz) equipped with I-3 filter block ideal for FITC fluorescence under UV light in the dark. Photographs were taken by Moticam Pro 285B.

Scanning electron microscopy (SEM)

Spores of *P. disseminata* were examined under scanning electron microscopy (SEM). Conidia of *P. disseminata* were sonicated separately under 35 MHz followed by washing five times in sterile distilled water,surface disinfected with 4% (w/v) chloramine-T and 300 ppm of streptomycin for 1 h, and then rinsed five times in sterile distilled water and were stored in Eppendorf's tube at room temperature. Each sample was placed within a separate aluminium disc cup (20 mm dia x 5 mm deep). Each sample was lifted from the bottom of the specimen dish with fine forceps and was positioned upright in a disc cup. The samples were then dried. All dried samples were mounted on double sided tape affixed to SEM specimen mounts and were subsequently sputter-coated with gold. Gold-coated samples were examined with a Philips 505 scanning electron microscope.

Transmission electron microscopy for immunogold localization of pathogen and defense enzymes in leaf and root tissues

Leaf and root samples (1-2 mm) of som plants (S5 morphotype) following artificial inoculation with *P. disseminata*, root colonization with *R. fasciculatus* and induced immunity by bioinoculants were excised in 0.1M sodium phosphate buffer pH 7.4 and immediately transferred to 2.5% glutaraldehyde in Eppendorf tubes for fixation at room temperature for 2-12 h. Dehydration was done in ascending grades of alcohol at intervals of 30 min at 4°C (30%, 50%, 70%, 80%, 90%) and two changes in absolute alcohol at 1 h interval each at 4°C in PLT-272(M) Fume Hood (Tanco). Infiltration was done twice in LR White resin (London Redin Co. Ltd) in absolute alcohol (1:1) for 1h each at 4°C. The samples were embedded in LR White and kept overnight at 4°C until further use. Before trimming, embedded samples were kept at room temperature for 3h, and a fresh change of LR white was done and kept at 56° C for 36 hrs. Moulds containing the samples

were roughly trimmed with a block trimmer (Reichert TM 60) fitted with a rotating milling cutter. A series of semithin sections of the selected blocks were cut with Belgium glass strips in microtome (Leica EM UC7), stained with 1% aqueous toluidine blue solution, and observed under a microscope. Ultrathin sections (60nm) were cut with fresh Belgium glass strips and picked up in nickel grids (100 mesh) for immunogold labeling. The grids containing ultrathin sections were floated in a blocking solution containing 2% skimmed milk agar for 30 min. Primary antibody (IgG raised against *P. disseminata* (fungal pathogen)/*R. fasciculatus* (AMF)/chitinase/glucanase) was diluted in 1% fish gelatin in the ratio of 1:20 and added. Grids were incubated with these IgGs separately for 24 hrs at 4°C and washed with drops (100 µl) of fish gelatin pipetted onto parafilm. Grids were then incubated with anti-rabbit IgG (whole molecule) gold antibody (secondary antibody) (Sigma-G7402) diluted in 1:5 in fish gelatin at room temperature for 3 hrs. Sections were stained with 2% uranyl acetate for 15 min, washed in double distilled water, post staining was done in 0.2% lead acetate for 5 min, and finally washed again in double-distilled water. Ultrastructural analysis of the section was performed with Morgagni 268D with TEM Imaging System. The specificity of labeling was assessed by the control test by incubating sections with rabbit pre-immune serum instead of the primary antibody.

RESULTS

Som plant (**Figure 1A**) is vulnerable to many foliar fungal diseases. Grey blight disease (**Figure 1B**) symptoms were noticed as appearance of small, oval and discoloured lesions scattered irregularly on young and mature leaves. In subsequent days following infection, brown or grey spots developed irregularly and in advanced stages the spots get collapsed, malformed and the entire leaf withered and dried off. Isolated fungal pathogen from infected leaf after completion of Kochs' postulate was identified as *Pestalotiopsis disseminata* based on 18S rDNA sequence which has been deposited in NCBI, GenBank database (accession no. KT697994).

Figure 1: A, Nursery grown healthy Som (*Persea bombycina*) plant showing; B, grey blight disease symptoms; C & D, Conidia of *Pestalotiopsis disseminata*, under (C) bright field microscope and (D) scanning electron microspcope

Mycelial growth and sporulation of *P. disseminata* was recorded. Large no. of conidia were produced within the acervuli. Conidial morphology was studied under bright field (**Figure 1C**) and Scanning electron microscope (**Figure 1D**). Conidia were long, fusiform, straight, rarely curved, 4-celled, slightly constricted at septa, concolourous median cells, apical and basal cells hyaline, apical cell cylindrical with 2 apical appendages (setulae) 25µm long, basal cell conical with a short basal appendage. Presence of relatively long apical appendages that are unbranched and unknobbed and attached to the tip of apical cells were observed.

Screening of resistance of *Persea bombycina* against *Pestalotiopsis disseminata*

Eight morphotypes (S1-S8) of well established pot grown som plants were inoculated with spore suspension of *P. disseminata* using whole plant inoculation technique and at 15, 20 and 30 days of interval following inoculation, appearance of disease symptoms were noted and percentage disease index (PDI) was calculated. Disease intensity was highest at each interval in S5 morphotype followed by S6, S7 and S2. It was least in S3 morphotype followed by S4, S1 and S8 (**Figure 2**).

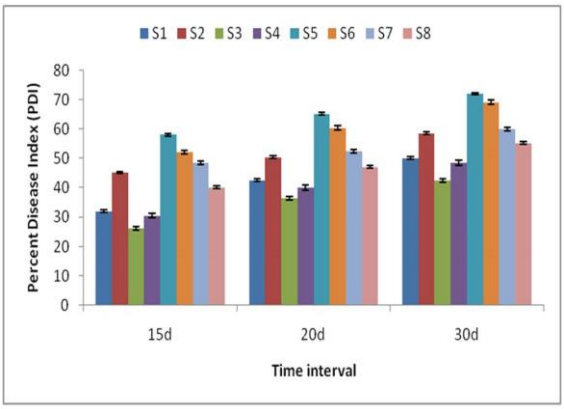

Figure 2: Screening of resistance of eight morphotypes of som plants against *P. disseminata* following whole plant inoculation

Immunodetection of *Pestalotiopsis disseminata* in som leaf tissues

Effectiveness of raising polyclonal antibodies (PAbs) against mycelial antigen preparations of *Pestalotiopsis disseminata* were checked by homologous cross reaction following immunodiffusion test. Strong precipitin reactions were observed. Control sets involving normal sera and pathogen (*P. disseminata*) antigen were negative. Purified IgG from PAbs of pathogen were further reacted with homologous antigen on nitrocellulose paper using dot immunobinding assay. Development of deep violet colour following homologous reaction with antigen and antibody

confirm its identity. Optimization of PAbs using plate trapped antigen coated enzyme linked immunosorbent assay (PTA-ELISA) format was done considering two variables, antigen and IgG concentrations. Doubling dilution of mycelial antigen of *P. disseminata* ranging from 40 to 0.312 µg/ml tested against IgG at a concentration of 40 µg/ml.ELISA values decreased with the decrease of antigen concentration but the values were still quite high indicating that the range of detection could be much lower. Leaf antigen of both healthy and infected samples of eight morphotypes were reacted with PAb of *P. disseminata* on nitrocellulose membrane. Development of violet colours of different intensities were noted in infected leaf samples. Deep violet colour was evident for S5 morphotype which was found to be highly suceptible in disease development following challenge inoculation. Further antigen preparation from both healthy and artificially inculated leaves of eight morphotypes were tested against 1:125 dilution of PAb raised against the pathogen using PTA-ELISA format. Results (Table 1) reveals that Absorbance (A_{405}) values for healthy leaf samples were significantly lower than corresponding infected samples. Highest absorbance value was evident in inoculated leaf sample of S5 morphotype.

Table 1: Detection offoliar fungal pathogen (*P. disseminata*) in artificially inoculated som leaves using DIBA and PTA-ELISA format

Leaf Antigen[a]	PAb of *P. disseminata*[b]			
Morphotype	Dot immunobinding assay (DIBA)[d]		PTA-ELISA values (A_{405})	
	Healthy	Inoculated[c]	Healthy	Inoculated[c]
S1	+	+ +	0.093±0.005	0.877±0.009
S2	+	+ +	0.089±0.021	0.932±0.014
S3	+	+ + +	0.084±0.008	0.899±0.008
S4	+	+ +	0.087±0.011	0.988±0.016
S5	+	+ + + +	0.092±0.005	1.342±0.018
S6	+	+ + +	0.098±0.003	1.022±0.006
S7	+	+ +	0.075±0.023	0.995±0.012
S7	+	+ +	0.086±0.009	0.887±0.007
S8	+	+ + +	0.799±0.005	0.892±0.009

[a]Antigen concentration – 40 µg/ml
[b]PAb dilution – 1:125
[c]48 h after inoculation with *Pestalotiopsis disseminata*
[d]NBT/BCIP used as substrate; Colour intensity of dots : + pink; ++ light violet; +++ violet; ++++ deep violet

Detection and localization of pathogen in som leaf tissues following indirect immunofluorescence using PAb of *Pestalotiopsis disseminata* labeled with FITC conjugates have also been done. Healthy leaf tissue exhibited autofluorescence as evident in cuticle (**Figure 3A**). Strong apple green fluorescence were observed in mesophyll tissues of grey blight infected leaf (**Figure 3 B,C**) of som plant (S5 morphotype). Conidia of *P. disseminata* showed bright apple green fluorescence throughout including setulae (**Figure 3 D,E**).

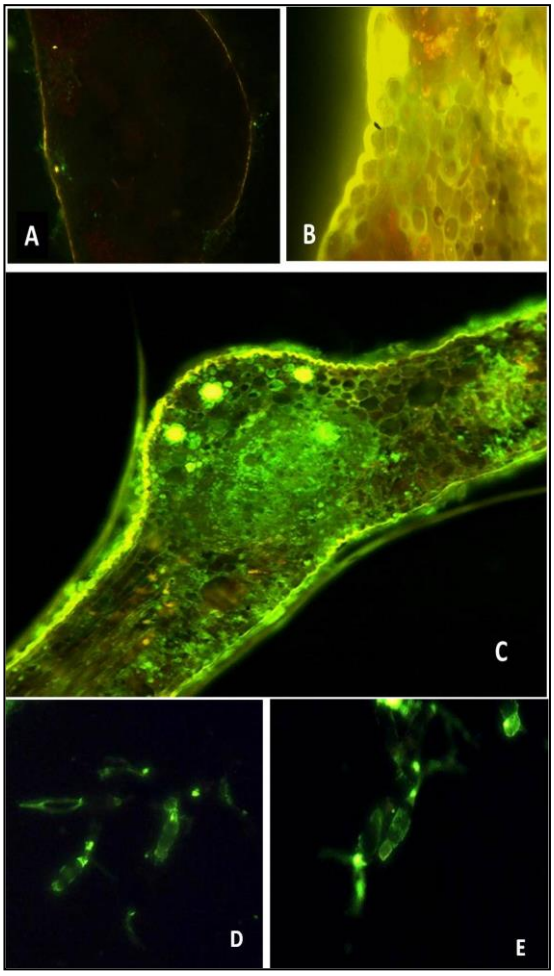

Figure 3: Transverse sections of A, healthy som leaf tissue showing autofluorescence on cuticle**;** B & C, grey blight infected som leaf; D & E, conidia of *Pestalotiopsis disseminata* treated with the PAb of *P. disseminata* and labeled with FITC conjugates showing apple green fluorescence.

In addition, immunogold localization of pathogen in leaf tissues of som plant (S5 morphotype) was carried out. Transmission electron micrographs of som leaf tissue infected with grey blight pathogen (*P. disseminata*) following reaction with PAb of the pathogen and immunogold labeling have showed deposition of gold particles predominantly localized in infected tissue (**Figure 4A,B**) indicating the regions where interaction has taken place and hence the pathogen location. The gold particles observed on the surface appeared either as individual spherical particles in an even distribution or as clusters of particles.

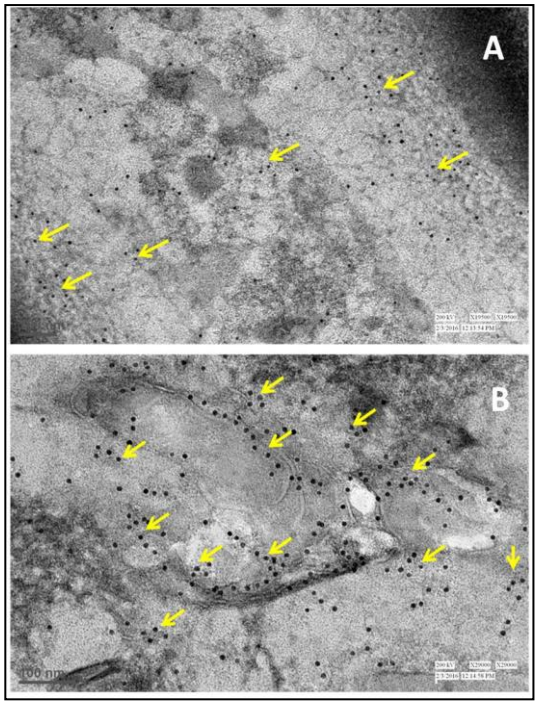

Figure 4: A & B, Transmission electron micrographs of a grey blight infected Som leaves probed with PAb of *Pestalotiopsis disseminata* and labeled with gold conjugates

In vitro evaluation of selected bioinoculants against *P. disseminata*

Potential isolate of PGPR (*Bacillus pumilus*) and PGPF (*Trichoderma asperellum*) which showed growth promotion in eight morphotypes of som plants have been tested *in vitro* for their antagonistic activity against grey blight pathogen (*P. disseminata*). Both of these isolates could inhibit the growth of the pathogen markedly (**Figure 5A-C**).

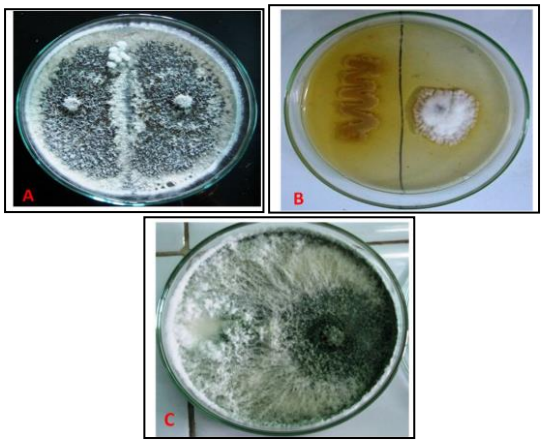

Figure 5: A, *In vitro* antagonistic test of PGPR and PGPF against *Pestalotiopsis disseminata*. *P. disseminata* alone; B, interaction with *Bacillus pumilus* (PGPR); C, interaction with *Trichoderma asperellum* (PGPF)

Activities of four important defense enzymes (POX, PAL, CHT and GLU), 48h following last application of PGPR from leaves revealed that activity of these enzymes increased following treatment in comparison to untreated control plants. Joint treatment of all bioinoculants yielded the best results than the other combinations in S5 morphotypes (**Figure 6**).

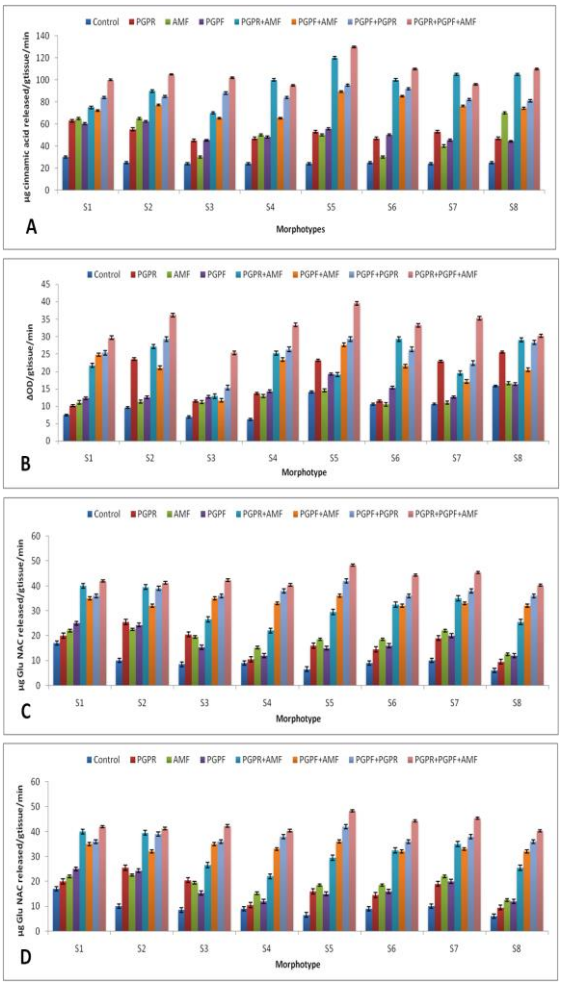

Figure 6: Activities of defense enzymes, A, PAL; B, Peroxidase; C, Chitinase; D, β,1-3 glucanase in control and treated som plants with bioinoculants (PGPR, AMF, PGPF) alone and in combinations.

Plant growth improvement status following application of Bioinoculants

Growth enhancement, in terms of height, no. of leaves and no. of branches were measured after 30 and 60 days of the last PGPR treatment. The results revealed that growth was significantly improved after each treatment in S5 morphotype after joint application of three bioinoculants. Growth promotion was almost 4 fold increased following treatments (**Table 2**).

Table 2: Observations on growth parameters of Som plants (S5 morphotype) at different intervals (days after inoculation)following application of bioinoculants in potted condition

Treatment	Height (cm)		No. of leaves		No. of branches	
	Days after inoculation					
	30	60	30	60	30	60
Control	26	31	9	32	3	3
PGPR	60	72	35	46	5	5
AMF	53	70	40	55	5	6
PGPF	54	71	41	57	6	7
PGPR+AMF	69	93	32	50	5	5
PGPF+AMF	69	73	34	53	7	6
PGPR+PGPF	61	92	36	59	9	8
PGPR+PGPF+AMF	64	93	53	73	11	13

PGPR (*Bacillus pumilus*), PGPF (*Trichoderma asperellum*), AMF (*Rhizophgus fasciculatus*)

Leaves from treated and untreated plants of S5 morphotypes were artificially challenge inoculated following detached leaf inoculation with spore suspension of *P. dissiminata* and percent lesion production was assessed 48, 72 and 96h of inoculation. Results revealed that approximately 48% reduction in disease development in S5 leaves of bioinoculants treated plants in relation to untreated control (**Table 3**). Assay of defense enzymes -PAL, POX, CHT and GLU in the leaves of the inoculated plants (S5) was carried out after every 24 h intervals up to 72 h. Enzyme activities increased following treatments and enhanced markedly after challenge inoculation with *P. disseminata* (**Figure 7A-D**).

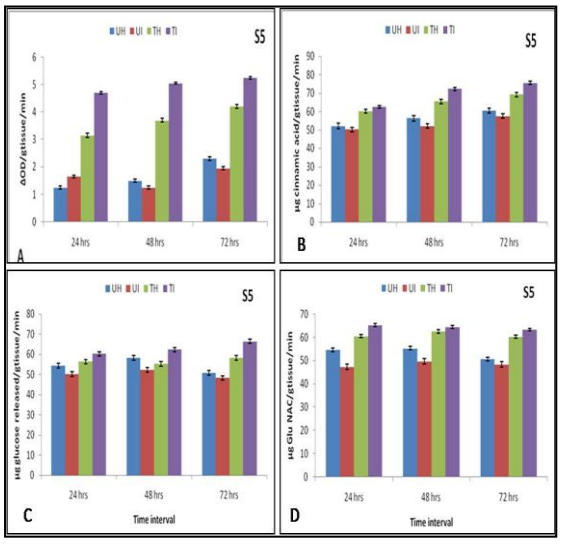

Figure 7: Changes in levels of defense enzymes in som plants (S5) following artificial inoculation with *P. disseminata.* A, Peroxidase; B, PAL; C, Chitinase; D, Glucanase

Table 3: Percent lesion production in som plants (S5 morphotype) after application with bioinoculants following inoculation with *P. disseminata*

Inoculated with *P.disseminata*	Percent lesion production[a] Hours after inoculation		
Treatment	48	72	96
Untreated inoculated	25.3±0.82	35.6±0.65	68.2±0.42
Treated inoculated			
PGPR+Pd	12.5±0.45 (12.8)[b]	20.3±0.35 (15.3)[b]	28.6±0.26 (39.6)[b]
AMF+Pd	18.5±0.84 (6.8)[b]	30.2±0.25 (5.4)[b]	39.6±0.45 (28.6)[b]
PGPF+Pd	16.2±0.42 (9.1)[b]	25.6±0.62 (10)[b]	36.5±0.22 (31.7)[b]
PGPR+AMF+Pd	12.3±0.15 (13)[b]	19.5±0.56 (16.1)[b]	25.5±0.52 (42.7)[b]
PGPF+AMF+Pd	11.2±0.16 (14.1)[b]	17.5±0.16 (18.1)[b]	22.5±0.32 (45.7)[b]
PGPR+PGPF+Pd	15.2±0.52 (10.1)[b]	15.3±0.28 (20.3)[b]	21.2±0.25 (47)[b]
PGPR+PGPF+AMF+Pd	10.3±0.65 (15)[b]	11.3±0.18 (24.3)[b]	20.2±0.48 (48)[b]

[a]Average of three separate trials (50 leaves inoculated for each treatment) [b]Parenthesis denotes percentage reduction in disease development in leaves of bioinoculants treated plants in relation to untreated control PGPR (*Bacillus pumilus*), PGPF (*Trichoderma asperellum*), AMF (*Rhizophgus fasciculatus*)
Pd (*Pestalotiopsis disseminata*)

Immunolocalization of AM Fungi in Som root tissue

Root colonization of som plants (S5) with *Rhizophagus fasciculatus* initially confirmed (**Figure 8A**). PAbs of *R. fasciculatus* and goat antisera specific to rabbit globulins conjugated with FITC were used for indirect immunofluroscence study to confirm root colonization of som plants with AM Fungi. On observation under fluorescence microscope using I3 UV filter bright apple green fluorescence of the hyphae, vesicles and arbuscules within the host tissue were evident. Fluorescence was more prominent towards the cortex layer in most of the tissues confirmed their successful colonization in som root tissue (**Figure 8B**). An indirect immunofluorescence test of AM fungal spores revealed a bright apple green fluorescence distributed throughout the spore wall (**Figure 8C**). Subtending hyphae also gave apple green fluorescence. Spores with their hyphae were more prominent in the root rhizosphere. Further immunogold localization of AMF in root tissues were confirmed by transmission electron microscopy using PAb of *R. fasciculatus* and labelled with antirabbit-IgG (whole molecule) gold conjugate (10nm). Gold particles were concentrated mostly near the cell wall and interfacial matrix (**Figure 8D**). Presence of gold particles proved successful colonization of *R. fasciculatus* in som

root tissues leading towards induction immunity in plants.

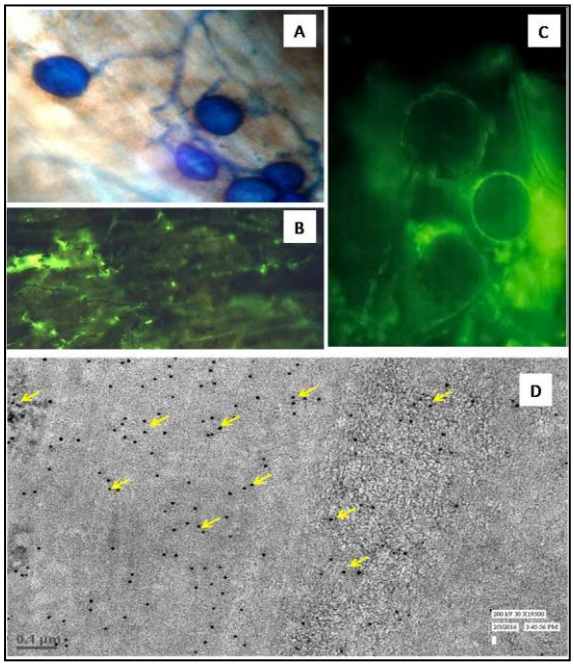

Figure 8: A, Root colonization of som plant (S5) with *R. fasciculatus* showing branched vesicles; B & C, Indirect immunofluorescence of som root tissue colonized with AM fungi treated with PAb of *R. fasciculatus* and labeled with FITC conjugates showing bright apple green fluorescence of (B) hyphae and (C) spores; D, Transmission electron micrograph of som (S5) root tissue following induction of immunity by AM Fungi treated with PAb of *R. fasciculatus* and labeled with antirabbit-IgG (whole molecule) gold conjugate

Cellular localization of glucanase and chitinase in leaf and root tissues of som plant following induction of resistance using bioinoculants

Enhanced activities of defense enzymes such as peroxidase, phenylalanine ammonia lyase, chitinase and glucanase as well as their time course accumulation have also been investigated following application of PGPR as well as AMF and PGPF in S5 morphotype. Induction of defense enzymes in som plants following treatment of bioinoculants has already been established. Glucanase and chitinase being an important PR protein related to induction of systemic resistance in plants, cellular localization of these enzymes in root and leaf tissue were studied.

Indirect Immunofluorescence

Cellular localization of glucanase and chitinase enzyme in leaves and roots of som plants following induction of resistance by bioinoculants (AMF, PGPF, PGPR) against *P. disseminata* were determined following indirect immunofluorescence test using PAb raised separately against glucanase and chitinase and labeled with FITC conjugates. Bright apple green fluorescence was observed in treated leaf and root tissues. Positive reactions with PAb of glucanase labeled with FITC on cortical tissues in som root (**Figure 9A**), while on mesophyll tissues (**Figure 9B**) in leaf are evident.

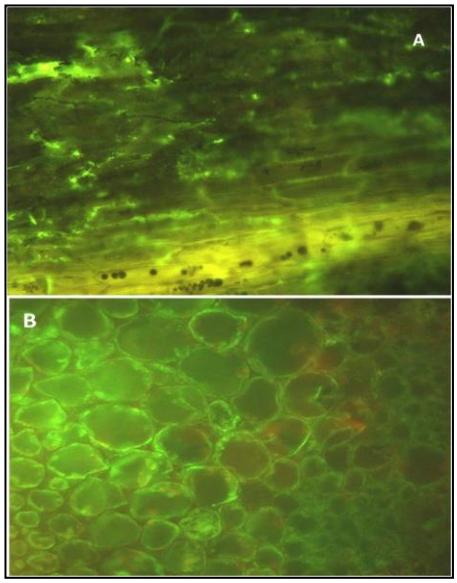

Figure 9: Cellular localization of glucanase in som (A) root and (B) leaf tissues following treatment with bioinoculants, probed with PAb of glucanase and labeled with FITC conjugate.

Similarly, bright apple green fluorescence was observed in som (S-5 morphotype) plant following induced immunity by bioinoculants (AMF, PGPF, PGPR) both in treated leaf tissue (**Figure 10A**) and root tissues (**Figure 10B**) probing with PAb of chitinase and labeled with FITC conjugate.

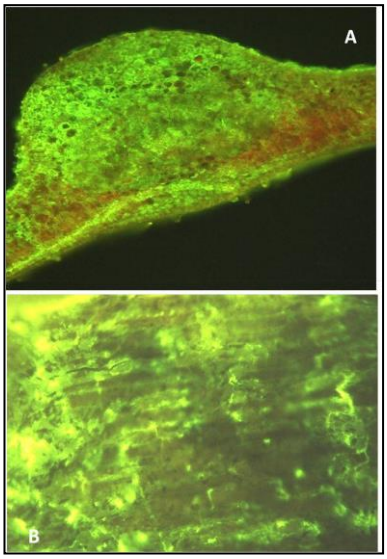

Figure 10: Cellular localization of chitinase in som (A) leaf and (B) root tissues following treatment with bioinoculants, probed with PAb of glucanase and labeled with FITC conjugate.

Immunogold localization

The accumulation of β-1,3 Glucanase and chitinase was further investigated in LR embedded cross sections of root and leaf tissues of som plant (S-5 morphotype) following induced immunity by bioinoculants using PAbs of glucanase and chitinase separately labeled with antirabbit-IgG (whole molecule) gold conjugate (10nm). Transmission microscopical observations revealed intense gold labelling corresponding to glucanase (**Figure 11 A,B**) and chitinase (**Figure 12A,B**) deposition in root and leaf tissues of induced plants. Gold labelling in the sections showed a high amount of labelling in chloroplast (**Figure 12A**) and host cytoplasm (**Figure 11B, 12A,B**).

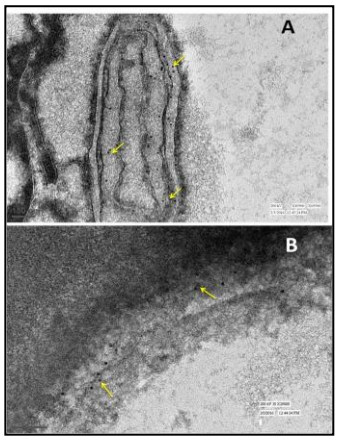

Figure 11: Transmission electron micrographs of (A) leaf and (B) root tissues of som plant (S5) probed with PAb of glucanase and labeled with gold conjugates following application of bioinoculants (*Rhizophagus fasciculatus*, *Bacillus pumilus and Trichoderma asperellum*) and challenge inoculation with *Pestalotiopsis disseminata*

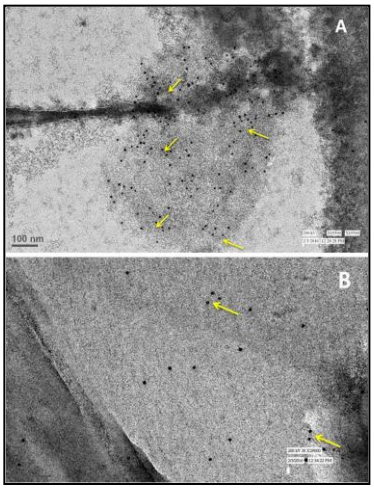

Figure 12: Transmission electron micrographs of (A) leaf and (B) root tissues of som plant (S5) probed with PAb of chitinase and labeled with gold conjugates following application of bioinoculants **(***Rhizophagus fasciculatus*, *Bacillus pumilus* and *Trichoderma asperellum***)** and challenge inoculation with *Pestalotiopsis disseminata*

This confirms the induction of glucanase and chitinase defense enzymes following application of bioinoculants **(***Rhizophagus fasciculatus*, *Bacillus pumilus and Trichoderma asperellum*) in som plants (S5 morphotype) and challenge inoculation with *Pestalotiopsis disseminata.*

DISCUSSION

Early detection of phytopathogens and diagnosis of plant diseases using serological and molecular techniques (Chakraborty and Chakraborty, 2021) have opened up a step in developing management strategies which leads towards sustaining crop production. Some of these rapid serological techniques are enzyme linked immunosorbent assay (ELISA), dot immunobinding assay (DIBA) and indirect immunofluorescence (IIF). These techniques have been used to detect various foliar fungal pathogens viz. *Exobasidium vexans* (Chakraborty and Sharma, 2007); *Glomerella cingulata* (Chakraborty *et al.*, 2008a); *Corticium invisum* (Chakraborty and Das Biswas, 2008); *Bipolaris sorokininana* (Chakraborty *et al*., 2016); *Drechslera oryzae* (Khati and Chakraborty, 2019); *Alternaria alternata* (Das Biswas and Chakraborty, 2020) and *Curvularia lunata* (Acharya *et al*., 2021) as well as fungal root pathogens viz. *Fomes lamaoensis* (Chakraborty *et. al.*, 2002a); *Ustulina zonata* (Chakraborty *et al.*, 2002b) *Macrophomina phaseolina* (Chakraborty *et al.*, 2012) and *Sclerotium rolfsii* (Bhagat and Chakraborty, 2020). In the present study, polyclonal antibody based immunodetection of *Pestalotiopsis disseminata* was developed using various immunological formats for early detection from leaf tissues in order to develop management strategies of grey blight disease of som plant. In this communication, we have first time demonstrated the immunogold localization of grey blight pathogen (*P. disseminata*) in som leaf tissue using IgG of the pathogen. Earlier immunogold labeling in wheat leaf infected with spot blotch pathogen (*Bipolaris sorokinana*) using PAb raised against pathogen has been reported by Chakraborty *et al*. (2016).

AM fungi utilization is highly encouraged in the modern global agricultural system, and their implication for biotic stress tolerance has been documented (Begum *et al*., 2019). Molecular detection of AM fungi and their role in symbiosis and crop protection (Chakraborty, 2019) and the efficacy of AM fungi as a vital component of sustainable crop production systems and its prospective for exploitation as an on-farm agro-put (Rodrigues and Rodrigues, 2020) have been reviewed. In recent years, the diversity of AM fungi in tea plants (Das Biswas *et al.*, 2020), *Citrus* spp. grown in Darjeeling hills (Allay *et al.*, 2021) and the mangrove ecosystem (Rodrigues, 2022) have also been reported. Inoculation with AM Fungi not only increases plant growth and offers

protection against soil-borne pathogens like *Fusarium solani* (Allay and Chakraborty, 2010), *Ustulina zonata* (Bhutia *et al*., 2012), *Sclerotium rolfsii* (Chakraborty *et al.,* 2016), but also improve root physiological activity, soil properties and fruit quality (Cao *et al.,* 2021; Cheng *et al.,* 2022).

AM fungi associated with *Persea bombycina* and their effect on improvement of plant health have been reported (Chakraborty *et al*., 2013). In the present communication, activation of defense response in som plants against *P. disseminata* following application of bioinoculants (*Rhizophagus fasciculatus*, *Bacillus pumilus* and *Trichoderma asperellum*) have been demonstrated. Application of bioinoculants singly or jointly increased plant growth, reduced disease severity and enhanced accumulation of peroxidase, phenyl alanine ammonia lyase, chitinase and glucanase (defense enzymes) following challenge inoculation with *P. disseminata.*

Induction of both systemic and local resistance in several crops such as cotton, rice, maize, tobacco, bean and lettuce by *T. harzianum* and in other pulses, cereals, plantation and horticultural crops by *T. asperellum* (Chakraborty *et al*., 2020) and utilization of plant growth promoting rhizobacteria (PGPR) with multiple beneficial traits for crop improvement (Chakraborty and Chakraborty, 2022) have been extensively worked out. Recently immunodetection of *Rhizophagus fasciculatus* and *Gigaspora gigantea* in soil and root tissues in *Citrus reticulata*, their exploitation as bioinoculants and cellular localization of defense enzymes following induced immunity against *Fusarium solani* have also been documented (Chakraborty and Allay, 2022). Cellular localization of defense enzymes by indirect immunofluorescence and immunogold localization of glucanase and chitinase by transmission electron microscopy confirms the induction of immunity in root and leaf tissues of som plant (S5 morphotype) against *P. disseminata* following application of bioinoculants.

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