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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 developed against *Fusarium solani*

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ABSTRACT

Two dominant arbuscular mycorrhizal (AM) fungi *Rhizophagus fasciculatus* and *Gigaspora gigantea*, their colonization with root tissues in *Citrus reticulata* along with their scanning electron microscopic views have been presented. Immunological formats for the detection of these two AM fungi were developed. IgG raised against *R. fasciculatus* and *Gi. gigantea* were used for immunodetection of AM fungal spores in soil labeled with FITC conjugates following an indirect immunofluorescence test. AM fungal spores showed a bright apple green fluorescence which was distributed throughout the spore wall. Subtending hyphae also gave apple green fluorescence. Spores with their hyphae were more prominent in the rhizosphere. Ultrathin sections of AM fungal colonized root stained with toluidine blue confirmed the presence of fine arbuscule branches within the root cells. Immunogold localization of AM fungi in mandarin roots was demonstrated. The gold particles were mostly concentrated near the cell wall. *R. fasciculatus* and *Gi. gigantea* were tested singly and in combination for their effect in inhibiting root rot of mandarin seedlings caused by *F. solani* in field conditions. Joint inoculation with AMF could effectively reduce disease incidence, correlated with increased accumulation of defense enzymes such as chitinase, β-1,3-glucanase, peroxidase, and phenylalanine ammonia-lyase. Cellular localization of chitinase in mandarin root and leaf tissues have been demonstrated following indirect immunofluorescence test using PAb raised against chitinase and labeled with FITC. Immunogold localization of chitinase following immunity induced by AM fungi in mandarin plants against *F. solani* confirmed the immunofluorescence results. It precisely showed the sites of chitinase expression as intense black gold particles distributed throughout the cell structure in mandarin roots.

Keywords: Plant immunity, Mandarin, AM fungi, Defense enzymes

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi have a 90% symbiotic association with higher plants, which are being commercially exploited as bioinoculants to improve plant growth (Chakraborty and Chakraborty, 2012). Arbuscules, vesicles, and hyphae in the roots and spores with hyphae in the soil rhizosphere are formed by AM fungi. Molecular detection of AM fungi and their role in symbiosis and crop protection have been documented (Chakraborty, 2019). 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). 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 has been reviewed by Rodrigues and Rodrigues (2020). In recent years, the diversity of AM fungi of tea plants (Das Biswas *et al.,* 2020), *Citrus* spp. grown in Darjeeling hills (Allay *et al.,* 2021), and the mangrove ecosystem (Rodrigues, 2022) have 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 and fruit quality (Cao *et al.,* 2021).

Mandarin (*Citrus reticulata*) is one of the most important fruit trees grown in the world. In India, mandarin grown in Darjeeling hills is in great demand as the fruit has a distinct flavour and is juicy. It is considered to be superior to Nagpur mandarin. The hyphal network of AM fungi with plant roots augments the roots in the soil surface, thereby causing an improvement in plant growth (Bowles *et al.,* 2016) as well as translocation of various nutrients (Rouphael *et al.,* 2015). The root hairs of citrus plants are very short, so they need mycorrhiza to help absorb adequate water and nutrition (Wu *et al.,* 2017). Effects of field inoculation with AM fungi and endophytic fungi on fruit quality and soil properties of New hall navel orange have been demonstrated (Cheng *et al.,* 2022).

In the present study, attempts have been made to develop immunoassays for the detection of two dominant AM fungi (*Rhizophagus fasciculatus* and *Gigaspora gigantea*) of *Citrus reticulata* in soil and root tissues along with their exploitation as bioinoculants for improvement of health status of mandarin plants and to determine the cellular localization of induced immunity developed against *Fusarium solani*, causing wilt of citrus.

MATERIALS AND METHODS

Isolation of AM fungi

AM fungal spores were isolated from rhizosphere soil of mandarin plant by wet sieving and decanting method (Gerdemann and Nicolson, 1963). The sucrose gradient centrifugation method (Daniels and Skipper, 1982), as illustrated by Allay *et al.* (2021), was used to purify AM fungal spores.

Microscopical observations of AM fungal spores

Clean spores were stained with Melzer's reagent and studied microscopically. The AM fungal spores were stored in Ringer's Solution at 15°C to 20°C or in sterile distilled water for further use. Identification of genera and species was done microscopically using the specific spore characters such as size, colour, shape, wall structure, surface ornamentation, and

bulbous suspensor by using identification manuals (Trappe, 1982; Schenck and Perez, 1990), standard keys (Walker, 1992), and website of INVAM (invam.ku.edu).

Single cell-line culture

To develop pure cell line culture of two specific AM fungi (*R. fasciculatus* and *Gi. gigantea*) following experimental setup was made using sterilized presoaked sorghum seedlings. Sorghum seeds were kept in the plate over a wet filter paper, and it was covered with black paper to avoid light. Within 96 hours, seeds germinated, and shoots developed from the open cut end. Selected AM fungal spores of *R. fasciculatus* and *Gi. gigantea* were carefully inoculated on the root surface and closed with black paper. Then 4-5 days after inoculation, the small seedlings were transferred in pots having sterile soil-sand mixture (50: 50). After 90 days, the desired spores of a single species were harvested. After harvesting, the same process was repeated thrice to get the desired spores and discard other entities. Once pure line culture was obtained, the same experiment was set up for mandarin seedlings. The inoculated seedlings were then maintained in a glass house in sterilized soil (**Fig. 1**). Colonization of mandarin roots with AM fungi was observed under a compound microscope following the protocol of Philips and Hayman (1970).

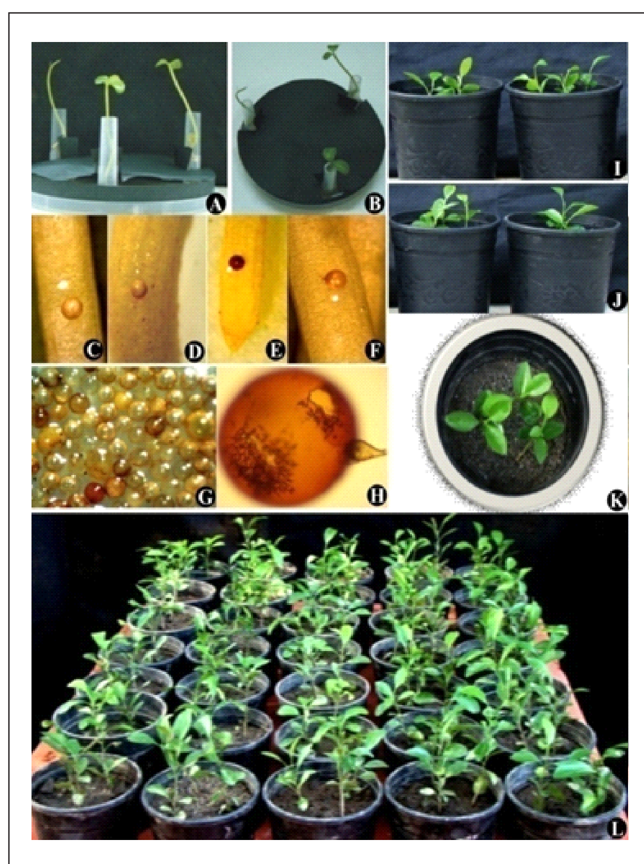

Fig. 1: Single spore inoculation of AM fungi in mandarin roots. (A-F) Experimental setup (G) Spores of *Gigaspora gigantea* (H) Single spore of *Gigaspora gigantea* (I-L) Maintenance of AM fungal inoculated seedlings of *Citrus reticulata* in sterilized soil and controlled condition in the glass house.

Disease assessment

The plants were inoculated with *Fusarium solani* following inoculum preparation and inoculation techniques as described by Chakraborty and Chakraborty (1989). Inoculated plants were examined at an interval of 10 days up to 30 days. Each time, the plants were uprooted, washed, and symptoms were noted. Finally, roots were dried at 60^{0}C for 96 h and weighed. Root rot index was calculated based on the percentage of root area affected, and they were graded into six groups, and a value was assigned to each group viz. 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering, and 20-40% of roots turn brown; 3- leaves withered and 50% of roots affected; 4- shoot tips also start withering; 60-70% roots affected; 5- shoots withered with defoliation of lower withered leaves, 80% roots affected; 6- whole plants die, with upper withered leaves remaining attached; roots fully rotted. The root rot index in each case was the quotient of the total values of the replicate roots and the number of roots (i.e., number of plants).

Extraction and assay of defense enzymes

Chitinase (E.C. 3.2.1.14) from healthy and AM fungal inoculated mandarin roots with or without challenge inoculation with the fungal pathogen was extracted in 0.1 M sodium citrate buffer, pH 5.0, and assayed following the method of Boller and Mauch (1988). The absorbance value was measured at 585 nm, and N-acetyl-D-glucosamine (GlcNAc) was used as standard. The enzyme activity was expressed as µg GLcNAc min^{-1} mg^{-1} fresh tissues. β-1,3-glucanase (E.C. 3.2.3.39) activity was assayed by the laminarin dinitrosalicylate method as suggested by Pan *et al.* (1991). Absorbance was recorded at 500 nm, and the enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh tissues. For peroxidase (E.C.1.11.1.7), the plant tissues were macerated to powder in liquid nitrogen and extracted in 0.1 M sodium borate buffer (pH 8.8) containing 2mM β-mercaptoethanol under ice cold conditions. Peroxidase activity was assayed by monitoring the oxidation of O-dianisidine in the presence of H_2O_2 and expressed as the increase in absorbance at 460 nm g^{-1} tissue/min^{-1} (Chakraborty *et al.,* 1993). Extraction of PAL (E.C. 4.3.1.5) was also done by following the method of Chakraborty *et al.* (1993), and the enzyme activity at 290 nm was expressed as µg cinnamic acid produced in 1 min g^{-1} fresh weight of tissues.

Scanning electron microscopy (SEM)

Spores of *R. fasciculatus* and *Gi. gigantea* were examined under scanning electron microscopy (SEM). Selected AM fungal spores 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.

Preparation of AM fungal antigens, production of antisera, and purification of IgG

Isolated clean spores of *R. fasciculatus* and *Gi. gigantea* 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* and *Gi. gigantea* and immunized separately in New Zealand white male rabbits of approximately 2kg of body weight following the protocol described by Chakraborty and Saha (1994). Before immunization with AM fungal antigen or chitinase, normal sera were collected from each rabbit. For developing antisera, intramuscular injections of 1ml AM fungal spore 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. Bleeding was performed by marginal ear vein puncture three days after the first six injections and then every fourth injection. Collected blood samples were incubated at 37^{0}C for 1hr 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^{0}C until required. Following the protocol of Clausen (1988), IgG was purified by DEAE-Sephadex column chromatography.

Indirect immunofluorescence

Purified IgG raised against spore antigens of *R. fasciculatum* and *Gi. gigantea* were used separately and labeled with goat antisera specific to rabbit globulins conjugated with fluorescein isothiocyanate (FITC) for indirect immuno-fluorescence study of isolated AM fungal spore, associated with rhizosphere as well as cellular location in AM fungal colonized root tissue of mandarin plants 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.

Transmission electron microscopy (TEM)

Mandarin root samples (1-2 mm) colonized with AM fungi 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 AMF/ chitinase) was diluted in 1% fish gelatin in the ratio of 1:20 and added. Grids were incubated with IgG of AM fungi 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

Successful colonization patterns of root samples of mandarin plants are presented in **fig. 2**. Extraradical hyphae (**Fig. 2A**) and spore (**Fig. 2B**), profuse arbuscules (**Fig. 2 C,D**), oval-shaped and flattened vesicles in abundance (**Fig. 2 E,F**) are evident. Both Arum and Paris type of hyphae were present that suggests colonization of both *Rhizophagus* and *Gigaspora* with root. Dark septate endophytes (DSE) were rarely present in the root cortex. Scanning electron microscopic observation was made of the two most dominant species viz. *R. fasciculatus* and *Gi. gigantea*. They showed differences in their wall characters and ornamentations. The basal attachments of these species showed distinct variations in their shape and size from one another (**Fig. 3 & 4**). Distinct bulbous suspensor of *Gi. gigantea* reveals the outer hyaline

layer and the conspicuous curved hyphal attachment (**Fig. 4 A,B**), whereas adhered hyphae of *R. fasciculatus* is evident with its sloughed and eroded outer hyaline layer covering the whole surface area.

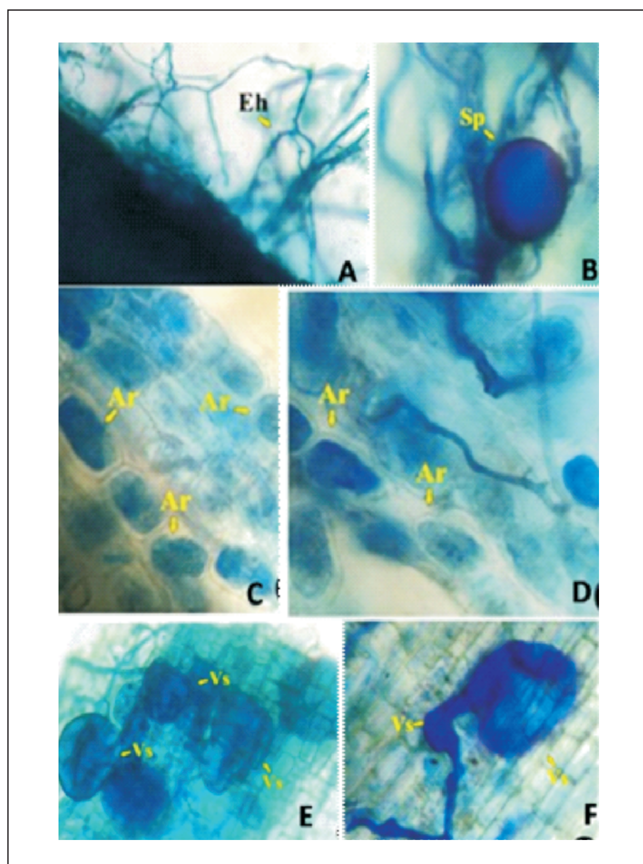

Fig. 2: Histopathological study of *C. reticulata* showing (A) extraradical hyphae, (B) extra-radical spore, (C&D) arbuscules (E) oval vesicles, and (F) flattened vesicle.

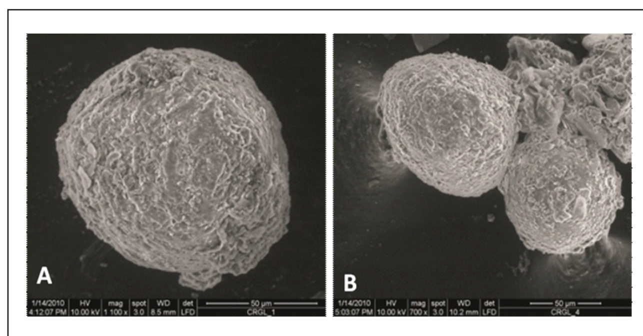

Fig. 3: (A&B): Scanning electron microscopic view of *Rhizophagus fasciculatus.*

Effect of *R. fasciculatus* and *Gi. gigantea* on growth promotion and disease suppression

Growth enhancement was evaluated in terms of a percent increase in height and leaf number in comparison to a similar increase in control plants. Increase in growth and number of

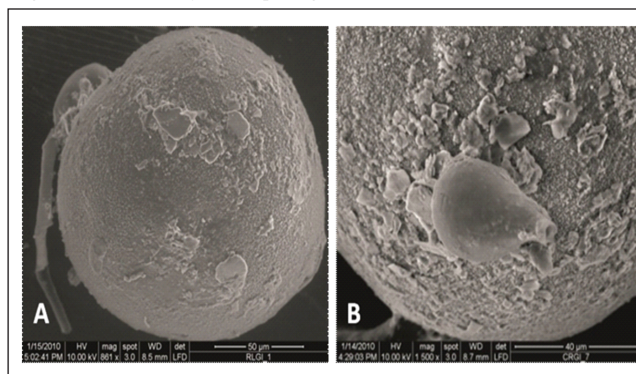

Fig. 4: (A&B) Scanning electron microscopic view of *Gigaspora gigantea* with close-up view of the bulbous suspensor (B).

leaves in mandarin plants inoculated with *R. fasciculatus* and *Gi. gigantea* alone was observed in relation to control. Growth enhancement was significantly higher when combination of both the AM fungi was applied. In the second set of trials, *R. fasciculatus* and *Gi. gigantea* were tested singly and in combination for their effect in inhibiting root rot of mandarin seedlings caused by *F. solani* in field conditions. Root colonization was confirmed by the histopathological study and our developed immunological formats prior to challenge inoculation with *F. solani*. *R. fasciculatus* and *Gi. gigantea* alone could effectively reduce disease incidence. However, combined inoculation with both *Gi. gigantea* and *R. fasciculatus* showed better results (**Table 1**).

Table 1: Effect of *R. fasciculatus* and *Gi. gigantea* on root rot disease development in mandarin plants

Treatments (Plants inoculated with)	Disease index Days after inoculation with *F. solani*		
	10	20	30
F. solani	2.0±0.577	3.9±0.057	5.8±0.057
Gi. gigantea+ *F. solani*	0.7±0.115	1.0±0.057	2.4±0.115
R. fasciculatus + *F. solani*	1.1±0.057	1.8±0.058	1.9±0.088
Gi. gigantea + *R. fasciculatus* + *F. solani*	0.4±0.057	0.9±0.057	1.6±0.057

Rot index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering, and 20-40% of roots turn brown; 3- leaves withered and 50% of roots affected; 4- shoot tips also start withering; 60-70% roots affected; 5- shoots withered with defoliation of lower withered leaves, 80% roots affected; 6- whole plants die, with upper withered leaves remaining attached; roots fully rotted. ± = SE.

Development of immunological format for detection of *R. fasciculatus* and *Gi. gigantea* in soil and mandarin root tissue

IgG raised against *R. fasciculatus* and *Gi. gigantea* were used for immunodetection of AM fungal spores in soil and their colonization in root tissue of mandarin plants using FITC conjugates and format of indirect immunofluorescence. Roots of mandarin seedlings successfully colonized with AM fungi were carefully separated, cleaned, and isolated. Purified

AM fungal spores from the rhizosphere of mandarin plants were also used in this experiment to develop the serological format for *R. fasciculatus* and *Gi. gigantea* for their cellular localization in root tissues of manadarin plants. AM fungal spores showed a bright apple green fluorescence distributed throughout the spore wall (**Fig. 5 A-C**). Subtending hyphae also gave apple green fluorescence. Spores with hyphae were more prominent in the root rhizosphere (**Fig. 5B**). Single spore of *Gi. gigantea* and aggregate of *R. fasciculatus* clearly showed the apple green fluorescence labeling with FITC. It was observed that the fluorescence was more intense on young spore walls. It was noticed that spores were distributed throughout the length of the root, including the tips of the root, indicating how quickly AM fungi colonize in the root cells. The hyphal proliferation within the feeder root tissues of mandarin plants was also specifically located with the help of FITC. On observation under a UV- microscope, bright apple-green fluorescence of the hyphae, vesicles, and arbuscules within the host tissue was also evident. Fluorescence was more prominent towards the cortex layer in most of the tissues, which was further confirmed in ultrathin sections of AM fungal colonized root stained with toluidine blue (**Fig. 6**), which showed the presence of fine arbuscule branches within the root cells. Immunogold localization of AM fungi in mandarin roots using IgG raised against *R. fasciculatus* and

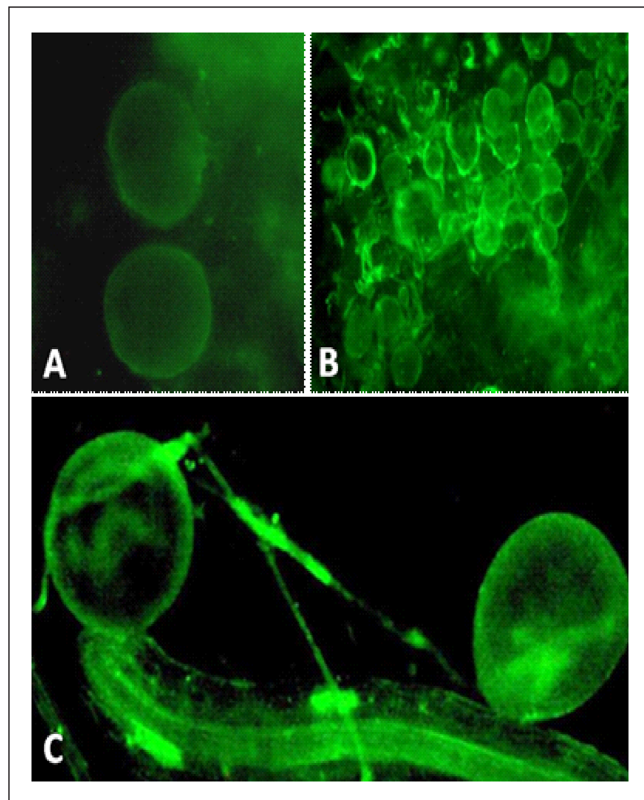

Fig. 5: (A-C) Indirect immunofluorescence of AM fungal spores of *R. fasciculatus* (A&B) and *Gi gigantea* (C) reacted with homologous IgG and labeled with FITC

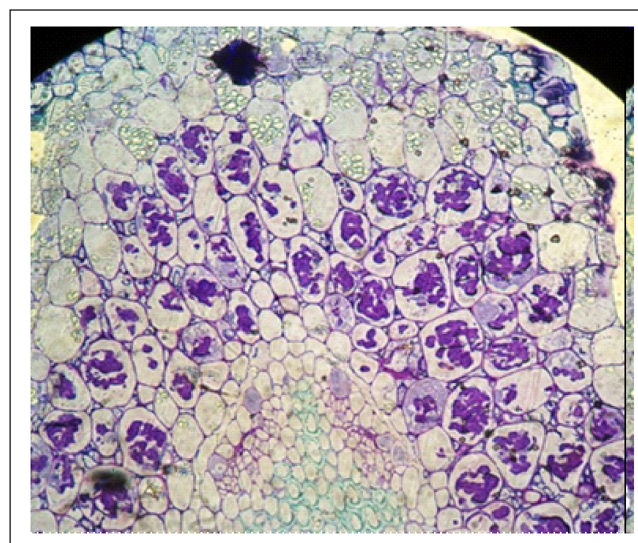

Fig. 6: Ultrathin section of AM fungal colonized mandarin roots stained with toluidine blue

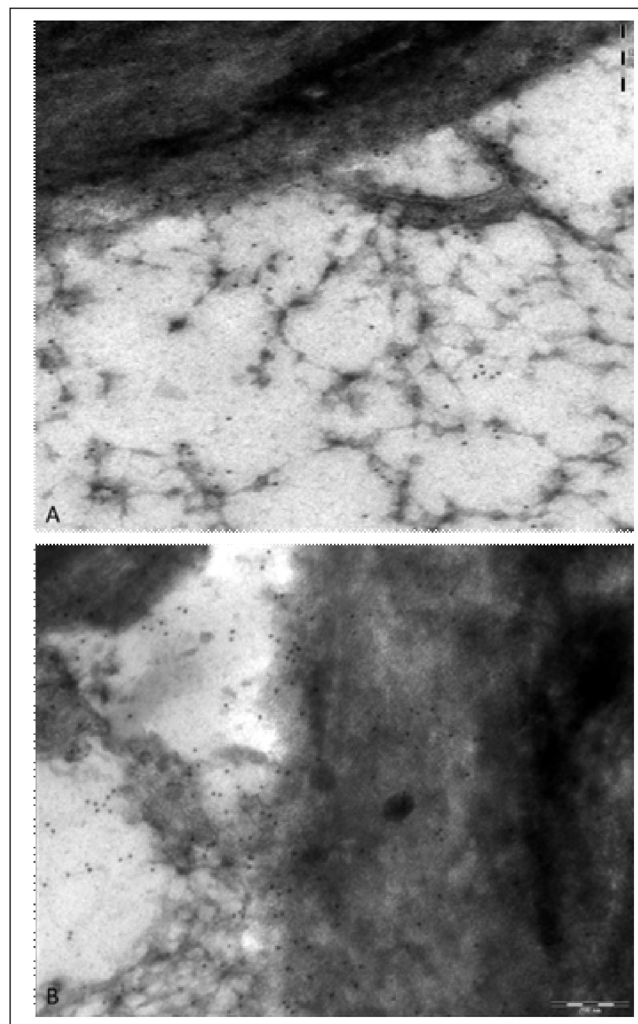

Fig. 7: Transmission electron micrographs showing immunogold localization of AM fungal colonized mandarin roots using IgG raised against *R. fasciculatus* (A) and *Gi. gigantea* (B).

Gi. gigantea has been demonstrated (**Fig. 7 A, B**), which were detected by the presence of black precipitates of colloidal gold. The gold particles were predominantly observed on the surface and appeared as either individual spherical particles or were found in closely associated groups or clusters of particles. Gold particles were mainly concentrated near the cell wall.

Biochemical changes in defense enzymes following induced resistance using bioinoculants

Significant biochemical changes were observed on application of *R. fasciculatus* and *Gi. gigantea* prior to pathogen challenge in mandarin plants in field conditions. Experiments were conducted to assess the effect of single and combined application of *R. fasciculatus* and *Gi. gigantea* on accumulation of defense enzymes in mandarin root and leaf tissue. Multifold increase in the activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonialyase in roots as well as in leaf of mandarin plants were observed after application of AM fungi followed by inoculation with *F. solani*. Overall results show that the defense enzyme activities were higher in leaves than in the roots (**Fig. 8**).

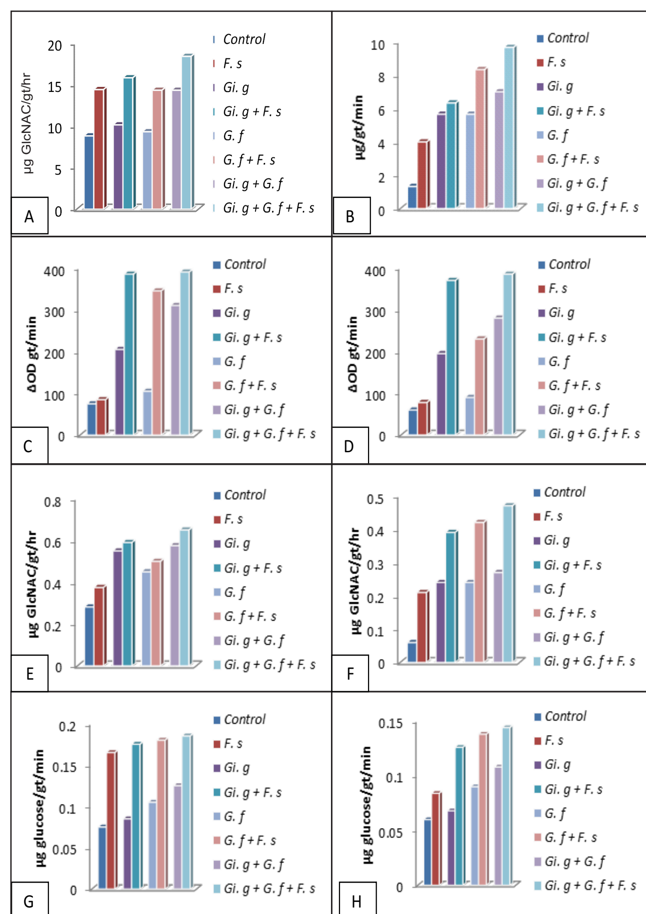

Fig. 8: PAL (A&B), peroxidase (C&D), chitinase (E&F), and glucanase (G&H) activities in AMF colonized leaf (A, C, E, & G) and root (B, D, F, & H) tissues of mandarin plants following challenge inoculation with *F. solani*.

Cellular location of chitinase in root and leaf tissues of *C. reticulata* following induction of resistance

Root rot incidence in mandarin plants was successfully reduced following successful colonization of AM fungi in root tissue prior to pathogen challenge. Disease reduction was brought about by enhanced activities of key defense enzymes like chitinase, glucanase, phenylalanine ammonia-lyase, and peroxidase, which increased significantly after pathogen challenge. Cellular localization of chitinase in mandarin root and leaf and tissues was determined following an indirect immunofluorescence test using PAb raised against chitinase and labeled with FITC. Root and leaf sections from untreated (control), untreated inoculated (with *F. solani*), AM fungal inoculated and AM fungal colonized root inoculated with *F. solani* were taken for immunolocalization of chitinase in AM fungal colonized as well as pathogen inoculated mandarin root and leaf tissues. Strong apple green fluorescence was observed in AM fungal colonized as well as pathogen

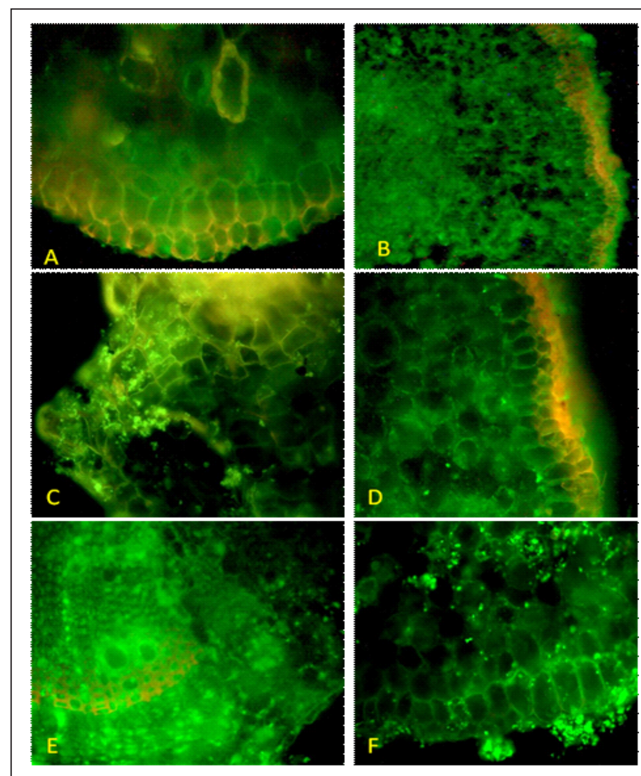

Fig. 9: (A-F) Cellular location of chitinase in mandarin roots colonized with AM fungi and challenge inoculation with *F. solani*. All the samples were probed with PAb of chitinase and labeled with FITC. (A) Untreated healthy root (B) root colonized with *R. fasciculatus* (C) root colonized with *Gi. gigantea* (D) root colonized with *Gi. gigantea* and inoculated with *F. solani* (E) root colonized with *R. fasciculatus* and inoculated with *F. solani* (F) untreated root inoculated with *F. solani*.

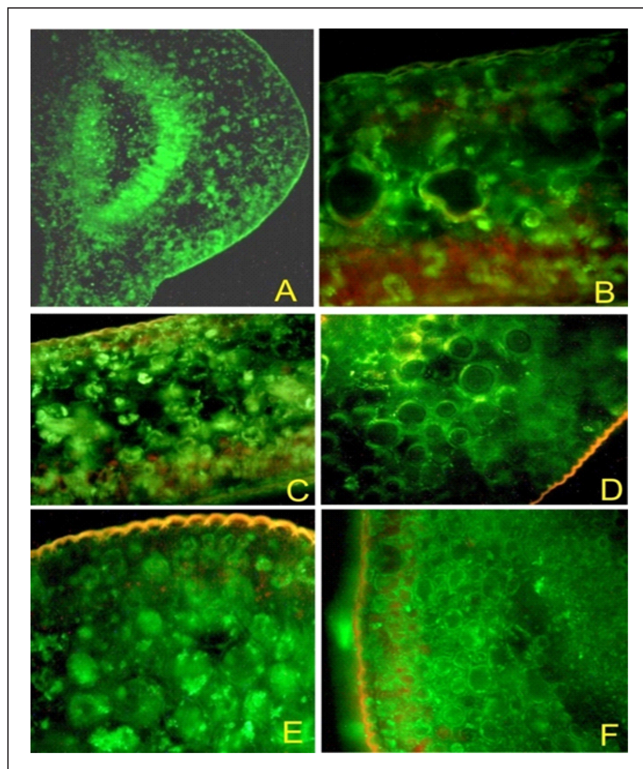

Fig. 10: (A-F) T. S of mandarin leaf tissues showing the cellular location of chitinase following root colonized with AM fungi and challenge inoculated with *F. solani*. All the leaf samples were probed with PAb of chitinase and labelled with FITC. (A) Plant colonized with *R. fasciculatus* (B) Untreated healthy leaf tissue, (C) Plant colonized with *Gi. gigantea* (D) Plant colonized with *Gi. gigantea* and inoculated with *F. solani* (E) Plant colonized with *R. Fasciculatus* and inoculated with *F. solani* (F) untreated plants inoculated with *F. Solani*.

inoculated roots (**Fig. 9 A-F**) and mesophyll tissues in leaf (**Fig. 10 A-F**) indicating induction of chitinase in root and leaf tissues.

Immunogold localization of chitinase following immunity induced by AM fungi in mandarin plants against *F. solani*

Immunogold labeling of AM fungal colonized roots either with *R. fasciculatus* or *Gi. gigantea* and challenge inoculated with *F. solani* was performed on ultrathin sections of LR-white embedded root tissues reacted with PAb raised against chitinase and labeled with antirabbit-IgG (whole molecule) gold conjugate (10nm). Control plant, in one set, was labeled with pre-immune serum instead of primary antibody, and another set was labeled with IgG of AM fungi. No gold labeling was observed in sections treated with pre-immune serum, whereas very weak gold labeling was observed on the cell wall of the control root treated with AM fungi. This indicates cross-reactive antigens (CRA) shared between AM fungi and mandarin root tissue and its cellular recognition is required for successful establishment in the soil. In both the sets, colonized with *R. fasciculatus* and *Gi. gigantea*, labeling was profuse (**Fig. 11A-F**), but gold labeling was more intense in roots colonized with *R. fasciculatus* and reacted with PAb of chitinase and labeled with antirabbit-IgG (whole molecule) gold conjugate (**Fig. 11 B, D**). Many clusters of particles were scattered around the cell wall. Gold particles were concentrated mainly near the cell wall and interfacial matrix. Intense black particles of gold were distributed throughout the cell structure in mandarin roots treated with AM fungi, followed by challenge inoculation with *F. solani* (**Fig. 11 E, F**). The immunogold localization study confirmed the immunofluorescence results and precisely showed the sites of chitinase expression.

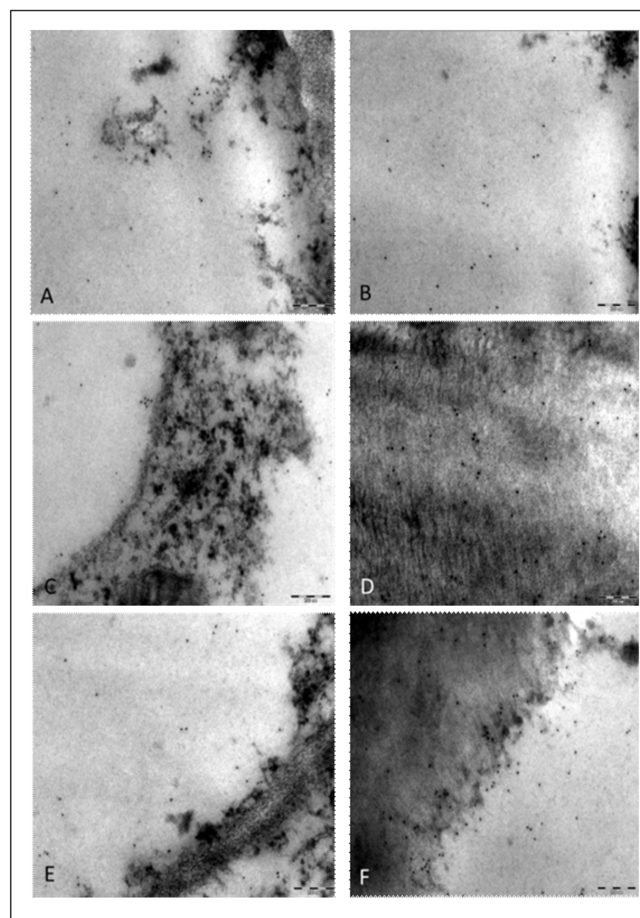

Fig. 11: (A-F) Transmission electron micrographs showing immunogold localization of chitinase in mandarin roots colonized with *Gi. gigantea* (A, C, & E), *R. fasciculatus* (B, D, & F) and challenge inoculation with *F. solani* (E&F) using PAb of chitinase labeled with antirabbit-IgG (whole molecule) gold conjugate.

DISCUSSION

Arbuscular mycorrhizal fungi provide a more suitable and environmentally acceptable alternative for sustainable agriculture and forestry (Adholeya, 2012; Chakraborty, 2019). The protective effect of AM fungal inoculation may be both systemic and localized. Diseases caused by fungal

pathogens persist in the soil matrix and residues on the soil surface. Damage to root tissue is often concealed in the soil. Thus, diseases may be noticed until the above-ground parts of the plant are severely affected. The diversity of AM fungi associated with root colonization of *Citrus reticulata* has been documented (Allay *et al.,* 2021). Colonization of the root by AM fungi generally reduces the severity of diseases caused by plant pathogens (Allay and Chakraborty, 2010, Bhutia *et al.,* 2012; Chakraborty *et al.,* 2016, 2019). The present work is aimed to develop a management strategy to control root rot of mandarin plants using two dominant AM fungi viz., *Rhizophagus fasciculatus* and *Gigaspora gigantea*. Spore wall morphology, as observed under scanning electron microscopy (SEM), revealed that spores of *R. fasciculatus* have a rough outer surface found in aggregates of 2-6, whereas the spore wall of *Gi. gigantea* was smooth-walled, having a typical bulbous suspensor.

Fluorescent antibody labeling with FITC is one of the best techniques (Chakraborty, 2021) to detect several microorganisms in soil and plant tissues (Chakraborty and Chakraborty, 2021). In addition, for the first time, we have developed immunological formats for immunodetection of these two AM fungi. IgG raised against *R. fasciculatus* and *Gi. gigantea*, reacted with spores and labeled with FITC conjugates. An indirect immunofluorescence test of AM fungal spores revealed a bright apple green fluorescence distributed throughout the spore wall. Subtending hyphae also gave apple green fluorescence. Spores with their hyphae were more prominent in the root rhizosphere. Ultrathin sections of AM colonized roots stained with toluidine blue also confirmed the presence of fine arbuscule branches within the root cells.

Combined inoculation with *Gi. gigantea* and *R. fasciculatus* could effectively reduce root rot disease incidence in mandarin plants, which was correlated with increased accumulation of defense enzymes such as chitinase, β-1,3-glucanase, peroxidase, and phenylalanine ammonia-lyase. Thus, protection in mandarin plants against *F. solani* has been achieved through induction of resistance due to an array of defense responses. One induced isoform of peroxizymes following dual application of *Glomus mosseae* and *Trichoderma hamatum* in *Citrus reticulata* has been confirmed in PAGE analyses (Allay and Chakraborty, 2010). Elicitation of chitinase, β-1, 3-glucanase, and peroxidase was also determined in tea plants following treatments with Josh-a bioformulations of AM fungi (Chakraborty *et al.,* 2007).

In the present study, we examined the systematic response of induced resistance in mandarin plants using PAb of chitinase following successful colonization with *Gi. gigantea* and *R. fasciculatus* and challenge inoculation with *F. solani*. Immunolocalization of chitinase in AM fungal colonized and pathogen inoculated mandarin root and leaf tissues were evident as strong apple green fluorescence in cortical root tissues and mesophyll leaf tissues, indicating induction of systemic resistance. Immunogold localization of chitinase was further confirmed as intense gold labeling over both cell walls and wall appositions, activating the defense response in the plants against root rot pathogen. Labeling of the plant cell wall was abundant at the point of penetration, where there was an accumulation of electron-dense material. A few gold particles were also seen in the parts of cell walls that adhered firmly to intercellularly growing fungal hyphae. Zuber *et al.* (2005) have illustrated the use of post-embedding immunogold labeling in ultrastructural pathology. In AM fungal primed date palm trees, accumulation of phenolic compounds could be correlated with the protection afforded against *F. oxysporum* (Jaiti *et al.,* 2007). Mycorrhiza could also protect plants against nematodes through the induction of resistance (Hao *et al.,* 2012). Resistance induced by AM fungi in rice against *Magnaporthe grisea* was dependent on the activation of genes involved in regulatory roles during host immunity and greater expression of defense effector genes during pathogen attack (Campos-Soriano *et al.,* 2012). Dual application of *Rhizophagus fasciculatus* and *Bacillus pumilus* (PGPR) led to enhancement in activities of defense-related enzymes, which were correlated with induced resistance in tea plants against *Sclerotium rolfsii* (Chakraborty *et al.,* 2016). Systemic induction of resistance by AM fungi has been proved by the fact that the defense responses are not restricted to the roots alone but are also evident in the shoots (Pozo *et al.,* 2010). It has also been reported that other than the systemic resistance induced by AM fungi in above-ground parts, the induction was also evident in neighbouring plants, probably due to the AM fungal hyphal network, which can travel long distances and pass on signals, thus acting as a plant to plant communicating system. Therefore, it is clear that bioinoculants induce immunity in different plants against phytopathogens (Chakraborty and Chakraborty, 2021). Observed plant health improvement and disease suppression against *F. solani* in mandarin plants using bioinoculants can be correlated with induced immunity in the host.

CONCLUSION

An efficient immunodiagnostic format has been developed for serological detection of *Rhizophagus fasciculatus* and *Gigaspora gigantea*, two dominant AM fungi of *Citrus reticulata* in soil and root tissues. Dual application of *R. fasciculatus* and *Gi. gigantea* have markedly reduced root rot disease of mandarin caused by *F. solani* and enhanced accumulation of defense enzymes. Induced systemic resistance (ISR) in mandarin plants against phytopathogen as evidenced by apple-green fluorescence in root and leaf tissues using indirect immunofluorescence test has further been confirmed by immunogold localization of chitinase in root tissues following application of these two dominant AM fungi and challenge inoculation with fungal pathogen.

REFERENCES

Adholeya, A. 2012. Mycorrhiza: A friendly association with plants towards sustainable food production. *J. Mycol. Plant Pathol.* **42:** 183-188.

Allay, S. and Chakraborty, B.N. 2010. Activation of defense response of mandarin plants against Fusarium root rot disease using *Glomus mosseae* and *Trichoderma hamatum*. *J. Mycol. Plant Pathol.* **40(4):** 499-511.

Allay, S., Thapa, K. and Chakraborty, B.N. 2021. Mycorrhizal association and root colonization of *Citrus reticulata*, *Citrus medica* and *Citrus limonia* grown in Darjeeling hills and foothills. *J. Mycopathol. Res.* **59(4):** 376-384.

Begum, N., Qin, C., Ahanger, M.A., Raza, S., Khan, M.I., Ashraf, M., Ahmed, N. and Zhang, L. 2019. Role of arbuscular mycorrhizal fungi in plant growth regulation: Implications in abiotic stress tolerance. *Front. Plant Sci.* **10:** 1068.

Bhutia L.P., Chakraborty B.N. and Chakraborty, U. 2012. Management of charcoal stump rot disease using AMF and PGPR in Temi Tea Estate, Sikkim. *J. Mycol. Plant Pathol.* **42(1):** 1-12.

Boller, T. and Mauch, F. 1988. Colorimetric assay for chitinase. *Meth. Enzymol.* **161:** 430-435.

Bowles, T.M., Barrios-Masias, F.H., Carlisle, E.A., Cavagnaro, T.R. and Jackson, L.E. 2016. Effects of arbuscular mycorrhizae on tomato yield, nutrient uptake, water relations and soil carbon dynamics under deficit irrigation in field conditions. *Sci. Total Environ.* **566:** 1223-1234.

Campos-Soriano, L., Garcia-Martiez, J., and Segundo, B.S. 2012. The arbuscular mycorrhizal symbiosis promotes the systemic induction of regulatory defense related genes in rice leaves and confers resistance to pathogen infection. *Mol. Plant Pathol.* **13(6):** 579-592; doi: org/10.1111/j.1364-3703. 2011.00773.x

Cao, M.-A., Wang, P., Hashem, A., Wirth, S., Abd_Allah, E.F. and Wu, Q.S. 2021. Field Inoculation of arbuscular mycorrhizal fungi improves fruit quality and root physiological activity of *Citrus*. *Agriculture* **11:** 1297; doi:org/10.3390/agriculture11121297.

Chakraborty, B.N. 2019. Arbuscular mycorrhizal fungi and mycorrhiza helper bacteria: molecular detection and their role in symbiosis and crop protection. *J. Bot. Soc. Bengal* **73:** 1-20.

Chakraborty, B.N. 2021. Immunotechniques. In: *Instrumentations Manual in Biology* (Eds.: Jyan Bikash Bhandari and Cyaria Gurung). Narosa Publishing House, New Delhi. pp.30-54.

Chakraborty, B.N. and Chakraborty, U. 2012. Molecular identification, recognition, cell defense responses and agricultural importance of AM fungi. *Rev. Plant Pathol.* **5:** 57-96.

Chakraborty, B.N. and Chakraborty, U. 2021. Molecular detection of fungal pathogens and induction of phytoimmunity using bioinoculants. *Ind. Phytopathol.* **74:** 307-322.

Chakraborty, B.N., Chakraborty, U., De, U. and Chakraborty, A.P. 2016. Induction of resistance in *Camellia sinensis* against *Sclerotium rolfsii* by dual application of *Rhizophagus fasciculatus* and *Bacillus pumilus*. *Arch. Phytopathol. Plant Prot.* **48 (13-16)**: 825-840; doi: org/10.1080/03235408.2016.1140607

Chakraborty, B.N., Chakraborty, U. and Allay, S. 2019. Wilt Root Rot Complex in Mandarin Plants and Activation of Defense against Pathogen. In: *Wilt Diseases of Crops* (Eds.: A.K. Bhattacharyya, B.N. Chakraborty, R.N. Pandey, D. Singh and S.C. Dubey) Indian Phytopathological Society and Today & Tomorrow's Publishers, New Delhi. pp. 293-321.

Chakraborty, B.N., Chakraborty, U., Barman, B.C., Bhutia, L. and Ghosh, P.K. 2007. Induction of Systemic Resistance in Tea Plants Against Root Rot Pathogens upon Field Application of VAM, Phosphate Solubilizing Fungus and Bacterium. In: *Rhizosphere Biotechnology/ Microbes in Relation to Plant Health: Retrospects & Prospects* (Eds.: Roy, A.K., Chakraborty, B.N., Mukadam, D.S. and Rashmi) Scientific Publishers (India), Jodhpur, pp. 61-71.

Chakraborty, B.N. and Saha, A. 1994. Detection and cellular location of cross-reactive antigens shared by *Camellia sinensis* and *Bipolaris carbonum*. *Physiol. Mol. Plant Pathol.* **44:** 403-416.

Chakraborty, U. and Chakraborty, B.N. 1989. Interaction of *Rhizobium leguminosarium* and *Fusarium solani* f. sp. *pisi* on pea affecting disease development and phytoalexin production. *Can. J. Bot.* **67:** 1698-1701.

Chakraborty, U., Chakraborty, B.N. and Kapoor, M. 1993. Changes in the levels of peroxidase and phenyl alanine ammonialyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. *Folia Microbiol.* **38:** 491-496.

Cheng, X.F, Xie, M.M., Li, Yan, Liu, B.Y., Liu, C.Y., Wu, Q.S, and Kuča, K. 2022. Effects of field inoculation with arbuscular mycorrhizal fungi and endophytic fungi on fruit quality and soil properties of New hall navel orange. *Appl. Soil Ecol.* **170:** 104-308.

Clausen, J. 1988. Immunochemical Techniques for the Identification of Macromolecules. In: *Laboratory Techniques in Biochemistry and Molecular Biology*. (Eds.: Burden, R.H. and Van Knippenberg, P.H.), Vol-1, Part III. pp. 64-65.

Daniels, B.A. and Skipper, H.D.1982. Methods for Recovery and Quantitative Estimation of Propagules from Soil. In: *Methods and Principles of Mycorrhizal Research* (Ed.: Schenck, N.C.). The American Phytopathological Society. pp. 29-36.

Das Biswas, R., De, U.K. and Chakraborty, B.N. 2020. Root colonization with arbuscular mycorrhizal fungi and dark septate endophytes in tea plants. *J. Mycopathol. Res.* **58 (1&2):** 51-55.

Gardemann, J.W. and Nicolson, T.H. 1963. Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. *Trans. Br. Mycol. Soc.* **46:** 235-244.

Hao, Z., Fayolle, L., Vantuinen, D., Chatagnier, O., Li, X., Gianinazzi, S. and Gianinazzi-Person, V. 2012. Local and systemic mycorrhiza-induced protection against the ectoparasitic nematode *Xiphinema index* involves priming of defense gene responses in grapevine. *J. Exp. Bot.* **63(10)**: 3657-3672; doi:org/10.1093/jxb/ers046

Jaiti, F., Meddich, A. and El Hadrami, I. 2007. Effectiveness of arbuscular mycorrhizal fungi in the protection of date palm (*Phoenix dactylifera* L.) against bayoud disease. *Physiol. Mol. Plant Pathol.* **71**: 166-173.

Ortas, I. 2012. Mycorrhiza in *Citrus*: Growth and Nutrition. In: *Advances in Citrus Nutrition.* (Ed.: Srivastava, A.) pp. 333-351.

Pan, S.Q., Ye, X.S. and Kuc, J. 1991. A technique for detection of chitinase, β-1,3 glucanase and protein patterns after a single separation using polyacrilamide gel electrophoresis or isoelectric focusing. *Phytopathol.* **8:** 970-974

Phillips, J.M. and Hayman, D.S.1970. Improved procedure for clear staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* **55:** 158-161.

Pozo, M.J., Jung, S.C., Lopez-Raez, J.A., and Azcon-Aguilar, C. 2010. Impact of Arbuscular Mycorrhizal Symbiosis on Plant Response to Biotic Stress: The Role of Plant Defence Mechanisms. In: *Arbuscular Mycorrhizas; Physiology and Function* (Eds.: Koltai, H. and Kapulnik, Y.), Springer, Netherland. Pp.193-207.

Rodrigues, B.F. 2022. Diversity of arbuscular mycorrhizal fungi in mangrove ecosystem A review. *J. Mycopathol. Res.* **60(2)**: 155-165.

Rodrigues, K.M. and Rodrigues, B.F. 2020. Arbuscular mycorrhizal (AM) fungi for sustainable agriculture. *J. Mycopathol. Res.* **58(3)**: 135-140.

Rouphael, Y., Franken, P., Schneider, C., Schwartz, D., Giovannetti, M. and Agnolucci, M. 2015. Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hort.* **196**: 91-108.

Schenck, N.C. and Perez, Y. 1990. *Manual for the Identification of VA Mycorrhizal Fungi* (3rd Ed), Gainesville, FL: Synergistic Publication, USA, pp. 286

Trappe, J.M. 1982. Synoptic keys to the genera and species of zygomycetous mycorrhizal fungi. *Phytopathology* **72**: 1102-1108

Walker, C. 1992. Systematics and taxonomy of the arbuscular endomycorrhizal fungi (*Glomales*), a possible way forward. *Agronomie* **12**: 887-897.

Wu, Q.S, Srivastava, A.K., Zou, Y.N. and Malhotra, S.K. 2017. Mycorrhizas in *Citrus*: Beyond soil fertility and plant nutrition. *Indian J. Agric. Sci.* **87(4)**: 427-423.

Zuber, C., Fan, J., Guhl, B. and Roth, J. 2005. Application of immungold labeling in ultrastructural pathology. *Ultrastruct. Pathol.* **29**: 319-330. doi.: org/10.1080/01913120590951310