Induction of Resistance in *Citrus reticulata* against *Fusarium solani* by Dual Application of AMF and *Trichoderma asperellum*

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Abstract

Decline of health status of nursery grown saplings of *Citrus reticulata* due to root rot caused by *Fusarium solani* is increasingly posing a major threat to the farmers of Darjeeling hill. One of the potential biocontrol fungus-*Trichoderma asperellum* isolated from mandarin rhizosphere, showing *in vitro* antagonistic reaction against the root rot pathogen-*F. solani* as well as showing plant growth promotion has been selected for application following root inoculation with *Glomus fasciculatum* and *Gigaspora gigantea,* dominant arbuscular mycorrhizal fungi (AMF) associated with mandarin roots. 18S rDNA sequence based molecular detection of *F. solani* and *T. asperellum* was done. PAbs separately raised against *F. solani*, *T. asperellum*, *G. fasciculatum* and *Gi. gigantea*, were purified and packaged into serological formats such as PTA-ELISA, DIBA, western blot and immunofluorescence. Successful root colonization with *G. fasciculatum* and *Gi. gigantea* was confirmed by their cellular localization in mandarin root tissues following FITC labeled immunofluorescence assay. Enhanced growth of the saplings in AMF inoculated plants was evident as compared with untreated healthy plants. Application of *G. fasciculatum*, Gi. *gigantea* and *T. asperellum* singly or jointly suppressed root rot of mandarin. Induction of major defense enzymes such as chitinase, β, 1-3 glucanase and peroxidase by treatment with AMF and *T. asperellum* was evident. One induced isoform of peroxizymes following AMF inoculation as well as application of *T. asperellum* was confirmed in PAGE analyses. Concomitant increase in defense enzymes following inoculation with *F. solani* was correlated with the induction of resistance in mandarin plants using bioinoculants.

1. Introduction

Fusarium solani is one the most notorious pathogen causing root rot in Darjeeling mandarin. It is prevalent in almost all orchards of Darjeeling hills and is one of the major reasons of heavy fruit loss due to decline of health of nursery grown plants. The beneficial effects of Arbuscular mycorrhizal fungi and biocontrol fungi either singly or dual application in root rhizosphere of plants have been proved (Davis et al., 1978; Allay and Chakraborty, 2010; El-Mohamedy et al., 2012). Our present investigation was to test the effects of *G. fasciculatum, Gi. gigantea* and *Trichoderma asperellum* on suppression of root rot disease and induction of resistance on *Citrus reticulata*.

2. Materials and Methods

2.1. Plant material

Mandarin (*Citrus reticulata*) seedlings (1 yr old) were obtained from Nirmaldass Orchard, Gurung Brothers Nursery, Baramangwa Busty, Darjeeling.

2.2. Isolation of AMF

AMF were isolated from rhizosphere of *C. reticulata* by wet sieving and decanting technique (Gerdemann and Nicolson 1963). Clean AMF spores were separated using the help of a simple microscope (20×), mounted on Polyvinyl-Lacto-Glycerol (PVLG), microscopical observations were made and photographs were taken. The spores were stored in Ringer's Solution at 4ºC or in sterile distilled water for further use.

2.3. Isolation of trichoderma sp. from mandarin rhizosphere

Trichoderma sp. was isolated from rhizosphere of *C. reticulata* in *Trichoderma* selective media (TSM) (Elad et al., 1980) *T. asperellum* were identified through National Center of Fungal Taxonomy, IARI, New Delhi and used for present investigation.

2.4. Fungal pathogen

Fusarium solani (Acc. No. 3719) causing root rot of mandarin was obtained from Culture collection of Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi.

2.5. Inoculation technique

F. solani was grown in sand-maize meal medium (maize meal: sand: water-1:9:1 w:w:v) in autoclavable plastic bags (sterilized at 20 lbs. pressure for 20 min) for a period of three weeks at 28ºC until the mycelia completely covered the substrate. Nursery grown mandarin seedlings were inoculated by adding 100 g of the prepared inoculum of *F. solani* to the rhizosphere soil.

2.6. Disease assessment

Disease assessment was performed following the method of Chakraborty et al. (2006) after 15, 30 and 45 d of inoculation.

2.7. In vitro studies

F. solani was paired with *T. asperellum* on solid medium as described by Chakraborty and Chakraborty (1989).

2.8. Inocula preparation and application of biocontrol fungi

2.8.1. AMF

Spores of *Glomus fasciculatum* and *Gigaspora gigantea* were separated by fine tweezers and needles under dissecting microscope. After washing with distilled water several times to remove the adhered debris they were separately inoculated in the roots of 7-10 d old seedlings of maize plants grown in black plastic pots (30 cm) having autoclaved soil. After 45 days, inocula were prepared by chopping roots of maize plants where extra radical spores of *G. fasciculatum* and *G. gigantea* were present. Approximately>175 spores 100 g^{-1} could be considered as potent inocula for application.

2.8.2. BCA

Inoculum of *T. asperellum* was prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28ºC for 10 days as described by Chakraborty et al. (2003).

2.9. Determination of growth of mandarin seedlings

Growth promotion was studied in terms of increase in height, number of leaves and biomass in potted plants. In each treatment, average of 20 replicate plants were taken and analyzed.

2.10. Extraction and quantification of soil phosphate

Soil sample (1g) was extracted as described by Mehlich, 1984 and quantitative estimation of phosphate was done as described by Knudsen and Beegle (1988).

2.11. Assay of enzyme activities

Leaves and roots of mandarin seedlings were collected for assay 72 h after inoculation. β-1,3-glucanase (E.C.3.2.1.39) was extracted and assayed from leaf samples following the method of Pan et al. (1991). Chitinase (E.C.3.2.1.14.) was extracted and assayed following the method of Boller and Mauch (1988). Peroxidase (E.C.1.11.1.7.) was extracted and estimated following the method described by Chakraborty et al. (1993).

2.12. Preparation of antigen

Antigens were prepared from mycelia of *F. solani* as well as from healthy and *F. solani* infected root tissues of mandarin plants following the methods as described by Chakraborty and Purkayastha (1983). They were stored at -20ºC and used as mycelial and root antigens.

2.13. Production and purification of polyclonal antibody

New Zealand white male rabbits were used to raise polyclonal antibodies against mycelial antigens of *F. solani* following the method of Chakraborty and Purkayastha (1983). IgGs were purified by DEAE-Sephadex column chromatography.

2.14. Immunodiffusion

Agar gel double diffusion tests were performed using PAb raised against *F. solani* following the method of Ouchterlony (1967).

2.15. PTA-ELISA

Plate trapped antigen-enzyme linked immunosorbent assay **(**PTA-ELISA) was performed essentially as described by Chakraborty and Sharma (2007).

2.16. Dot immunobinding assay

Mycelial antigens prepared from root pathogens of mandarin (*F. solani* and *F. oxysporum*), healthy and artificially inoculated (with *F. solani*) roots of *C. reticulata* were loaded on nitrocellulose membrane filters using Bio-Dot apparatus (Bio-Rad). Dot immunobinding assay was performed using PAb of *F. solani*.

2.17. Western blotting.

Protein samples were electrophoresed on 10% SDS-PAGE gels as suggested by Laemmli (1970) and electrotransferred to NCM using semi-dry Trans-blot unit (BioRad) and probed with PAbs of *F. solani*. Hybridization was done using alkaline phosphatase conjugate and 5-bromo-4-choloro-3-indolylphosphate (NBT-BCIP) as substrate. Immunoreactivity of the proteins was visualized as violet coloured bands on the NCM.

2.18. Immunofluorescence

PAbs of AMF, *F. solani,* and goat antisera specific to rabbit globulins conjugated with FITC were used for indirect immunofluorescence study following the method of Chakraborty et al. (1995). Observations were made using a

Biomed microscope (Leitz) equipped with an I3 filter block ideal for FITC fluorescence under UV light in the dark. Photographs were taken by Leica Wild MPS 48 camera on Kodak 800 ASA film.

2.19. Preparation of genomic DNA.

Extraction of genomic DNA from 5 days old mycelia of *F. solani* and *T. asperellum* were done as described by Ma et al. (2001). The quality and quantity of the DNA sample was analyzed spectophtometrically.

2.20. ITS-PCR amplification

The isolates of *Fusarium* and *Trichoderma* were taken up for ITS-PCR amplification.

3. Results and Discussion

AMF from rhizosphere soil of mandarin were initially screened. High population of *Glomus fasciculatum* and *Gigaspora gigantea* were observed and hence were selected for inoculation. Total phosphate content of soil was determined after application of *G. fasciculatum,* and *G. gigantea* singly or jointly. Soil phosphate content decreased after application of AMF indicating that the plant could uptake phosphorus which had been solubilized by AMF (Table 1).

Dot immunobinding assays confirmed the effectiveness of raising antibodies against *F. solani*. Previous studies have also suggested that common antigens may be indicators of plant host-parasite compatibility (Chakraborty, 1988). Absorbance values in PTA-ELISA were significantly higher for infected root extracts than for healthy controls up to a concentration of 2 mg L.

Mycelial antigen of *F. solani* was analysed on SDS-PAGE and then western blot analyses were done using homologous PAb (Table 2). Antibody labeling with fluorescein isothiocyanate (FITC) is a powerful technique used to determine the cell or tissue location of major cross reactive antigens (CRA) shared by host and parasite. Specific detection of cross reactive antigens was confirmed as bright green fluorescence in young hyphal tip as well as in conidia of the pathogen and in spores and hyphae of AMF. ITS region of rDNA was amplified using genus specific T/ITS1 & T/ITS4 (for *Trichoderma*) and Fcg 17 F & Fcg 17 FR (for *Fusarium*) primers. Amplified products of size in the range of 550-700 bp was produced by the all primers. The primer pairs Fcg 17 F and Fcg 17 R were found to be highly specific for *Fusarium* genus. Molecular techniques based on the polymerase chain reaction (PCR) have been used as a tool in genetic mapping, molecular taxonomy, evolutionary studies, and diagnosis of several fungal species (Williams et al., 1990, Clulow et al., 1991, Welsh et al., 1991 and McDonald, 1997). *Trichoderma* produced a single amplified product ranging from 600-620 bp. In order to identify one of our isolates of *T. asperellum* (RHS/M512) obtained from mandarin rhizosphere, 18S rRNA gene sequence which has been submitted to GenBank databases (Acc. No. HQ 265418) was compared and confirmed with other ten *Trichoderma* 18S rRNA gene sequences from NCBI database. Antibiosis to *F. solani* by biocontrol agent (*T. asperellum*) was evaluated *in vitro* and *in vivo*. The application of *T. asperellum* and AMF

Table 1: Soil phosphate content in rhizosphere of mandarin plants following root colonization with *G. fasciculatum* and *Gi. Gigantea*

Treatment	Soil phosphate (ug g^{-1} tissue)
Control	48.43
G. fasciculatum	35.11
Gi. gigantea	31.46
G. fasciculatum+Gi. gigantea	30.06

Table 2: ELISA and Dot-Blot values of reactions between PAbs of defense enzymes and enzyme extracts from treated mandarin plants

Antigen source*	PAb of chitinase		PAb of β□1,3-glucanase	
	A 405 ELISA	Colour intensity Dot-Blot	A 405 ELISA	Colour intensity Dot-Blot
Control	0.034	Light pink	0.049	Light pink
F. solani	0.036	Light pink	0.052	Light pink
F. solani+G. fasciculatum	0.499	Deep purplish	0.423	Dark pink
F. solani+Gi. gigantea	0.521	Deep purplish	0.368	Dark pink
F. solani+T. asperellum	0.487	Deep purplish	0.369	Dark pink
F. solani+G. fasciculatum+Gi. gigantea	0.768	Dark pink	0.445	Deep purplish
F. solani+G. fasciculatum+T. asperellum	0.745	Dark pink	0.525	Dark pink
F. solani+Gi. gigantea+T. asperellum	0.875	Dark pink	0.763	Deep purplish
F. solani+G. fasciculatum+Gi. gigantea+T. asperellum	0.980	Deep purplish	0.856	Dark pink

*Enzyme extracts from leaves of plants treated as mentioned

(*G. fasciculatum* and *Gi. gigantea*) to the soil as biocontrol agents, resulted in reduced disease severity and enhanced plant growth. Application of *G. fasciculatum, Gi. gigantea* and *T. asperellum* in the rhizosphere of *Citrus* plants gave most significant results in the growth of seedlings and numbers of leaves. Marked reduction in disease development was evident following triple inoculations of *G. fasciculatum, Gi. gigantea* and *T. asperellum*. Increased accumulation of defense enzymes

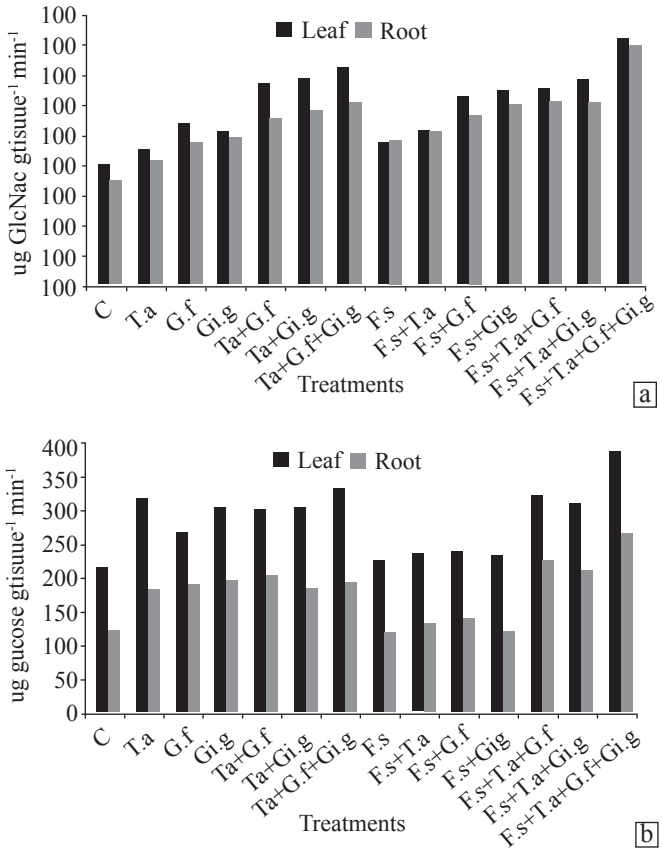

Figure 1: Chitinase (a) and Glucanase (b) activities following application of AMF and BCA

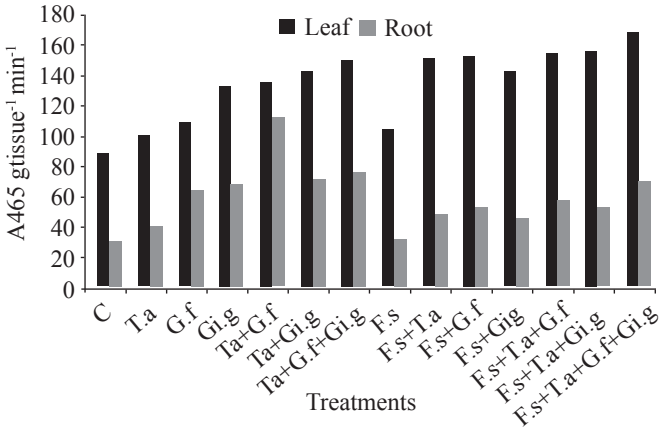

Figure 2: Peroxidase activities following application of AMF and BCA

such as chitinase (Figure 1a), β-1,3-glucanase (Figure 1b) and peroxidase (Figure 2) demonstrated defense responses of mandarin plants during early stages of root colonization.

Isoforms were observed on staining of peroxidase after native PAGE. A few of them were constitutively present in healthy roots and leaves. New isoforms were noticed in mandarin leaves following inoculation of roots with *G. fasciculatum, Gi. gigantea* and *T. asperellum,* singly or jointly. Increased activity of chitinase, β-1, 3-glucanase and peroxidase were also determined in mandarin plants following treatments with *G. mosseae* and *T. hamatum* (Allay and Chakraborty, 2010)

4. Conclusion

The present work mainly focused on developing a biological management strategy to control root rot and wilt complex of mandarin plants caused by *F. solani*. Results of present study indicate that *G. fasciculatum, Gi. gigantea* and *T. asperellum* could promote growth and suppress root rot of mandarin. Combined application of both AMF and BCA gave better results.

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