Research Article

Screening and Selection of Potential Plant Beneficial Rhizosphere Fungi from Medicinally Important Plant Species in Tamil Nadu, India

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Abstract

The problem of chemical fertilizers and pesticides can be solved by the action of plant growth promoting fungi (PGPF). The indigenous fungal isolates obtained from the rhizosphere soil can be used as they are said to solubilize the insoluble zinc, phosphorous, potassium etc. They are known to control the different fungal pathogens and thus promoting the plant growth and health. In the present study, attempt was made to isolate soil beneficial fungi from the rhizosphere soil samples of important medicinal plants such as *Aegle marmelos, Azardirachta indica* and *Citrus limon.* A total of 22 fungal isolates was isolated and screened for their efficacy on phosphate and zinc solubilization and antagonistic potential against selected plant pathogenic fungi under *in vitro* condition and the isolates were identified up to species level.

Keywords: Rhizosphere, antagonistic, *Aspergillus oryzae, Penicillium citrinum*, plant growth promoting fungi.

Introduction

Diseases cause major losses in nurseries and plantations resulting in failure of planting material. In nurseries, they cause heavy damage to seedlings and hence reduce both quantity and quality of planting stocks. In plantations, they cause major problems resulting in the reduction of biomass production or loss of germplasm collections. Thus, the economic loss resulting from nursery diseases are considerable. Therefore, raising disease free, healthy seedlings including medicinally valuable plants is not only important for maintaining a good nursery stock but also essential in establishing a healthy stand in the field for better productivity (Solaiman and Anawar, 2015). Different disease problems like damping-off, root rot, stem rot, seedling wilt, collar-rot etc. caused by various soil and root-borne pathogens and resulting heavy loss of quality seedlings in tree nurseries in India (Bakshi, 1971; Sharma *et al.,* 1985; Sankaran *et al.,* 1986; Mehrotra, 1990; Mohanan and Sharma, 1993; Mohan and Manokaran, 2001; Mohan *et al.,* 2002; Mohanan, 2008; Mohan and Manokaran, 2013; Mohan, 2016). Different soil and root-borne pathogens cause damage to seedlings of *Aegle marmelos, Azadirachta indica* and *Citrus limon* in nursery as well as in field conditions. Application of chemical fungicides was adopted to control different pathogens in nurseries and they are not safe. Integrating suitable control methods like use of beneficial microbes as bio-control agents and bio-fertilizers should be adopted in tree nurseries to produce healthy and quality planting stock (Kean *et al.,* 2010).

Plant growth promotion can also occur indirectly by control of pathogens (bio-control) via synthesis of antibiotics or secondary metabolite-mediated induced systemic resistance. Microbial colonization of the plant root surface is not uniform, but instead occurs in patches along the root, ultimately covering ~15-40% of the total plant root surface. The density and structure of the microbes on the root surface are dictated by nutrient availability and physico-chemical variations throughout the root surface. Root exudates can serve as a food source and chemo-attractant for microbes which then attach to the root surface and form micro colonies.

Common sites for bacterial attachment and colonization are at epidermal cell junctions, root hairs, axial groves, cap cells, and sites of emerging lateral roots (Danhorn *et al.,* 2007). Report on the status of beneficial soil fungi from the rhizosphere of medicinally important plants and their role as bio-control agents in controlling different plant pathogenic fungi are scanty. Hence, an attempt was made for isolation and identification of potential beneficial soil fungi from the rhizosphere of medicinally important plant species such as Indian Bael (*Aegle marmelos*), Neem (*Azadirachta indica*) and Lemon (*Citrus limon*) and also determining their plant growth promotion and bio-control efficacy against selected plant pathogens under *in vitro* condition.

Materials and methods

Collection and physico-chemical analysis of roots and soil samples: Roots and soil samples were collected from the rhizosphere region of selected medicinally important plant species such as Indian Bael (*Aegle marmelos*), Neem (*Azadirachta indica*) and Lemon (*Citrus limon*) grown in Forest Campus, R.S. Puram, Coimbatore, Tamil Nadu. All the sample were brought to the laboratory in zip lock poly bags, sealed tightly and immediately transported to laboratory and kept in refrigerator at 4°C until further use. All the soil samples were analyzed for various physico-chemical parameters in Soil and Water Testing Laboratory of IFGTB, Coimbatore by using standard procedures.

Isolation and enumeration of phosphate solubilising fungi from rhizosphere soil samples: About 1 g of each soil sample was weighed and suspended in 100 mL of sterile distilled water and mixed well. The suspension was then serially diluted up to 10^{-2} and 10^{-1} and 10^{-2} dilutions were used for plating (spread).The screened fungal isolates from spread plates were grown in Potato Dextrose Agar (PDA) plates individually and they were cultured in PDA slants, stored at 4°C for future use. The fungal isolates were stained by using cotton blue in lacto phenol to study the morphology and identify the organisms under microscope.

Estimation of population density of AM fungal spores from rhizosphere soil samples: AM fungal spores were isolated from the rhizosphere soil samples of all the plants by adopting wet sieving and decanting method (Gerdemann and Nicolson, 1963) and estimated population density.

Estimation of percent root colonization of AM fungi: The collected root samples were washed in tap water and immediately fixed in Formalin-Acetic Acid-Alcohol (FAA). The roots were analyzed by adopting root clearing technique (Phillips and Hayman, 1970) and the percentage of root colonization was estimated by using the following formula:

$$\text{Percent (\%) root colonization} = \frac{\text{Number of root segments colonized}}{\text{Number of root segments examined}} \times 100$$

Determination of phosphate solubilisation: In qualitative method, fungal colonies were grown in Pikovaskaya's Agar plates in a sterile condition by incubating for 72 h at RT. After that zone of clearance or hallow formation was observed, measured and recorded and Solubilization Efficiency (SE) was calculated by the formula given below:

$$\text{Solubilization Efficacy} = \frac{\text{Growth diameter}}{\text{Diameter of colony}} \times 100$$

In quantitative method, the fungal isolates were grown in Pikovaskaya's broth medium and then the 5 day old culture was centrifuged and the supernatant was collected. To 0.5 mL of supernatant, 0.9 mL of 5% sodium dodecyl sulphate, 1 mL of 1.25% ammonium molybdate solution in 2M HCl, and then 0.1 mL of 1 g/L ascorbic acid solution was added and mixed well. This mixture was incubated at RT for 30 minutes and the absorbance was read at 700 nm. Maximum phosphate solubilizing fungal isolates were selected for PGPF activities.

Determination of zinc solubilization: The commercial preparation of ZnO was supplemented to PDA for final concentration of 0.5% (w/v). The selected fungi were inoculated in ZnO amended plates and incubated at 25°C for 7 d. The solubilizing ability was assessed by the method proposed by Fomina *et al.* (2003), Martino *et al.* (2003) and Sutjaritvorakul *et al.* (2011).

$$\text{Solubilization Index} = \frac{\text{Mycelium diameter + Zone diameter}}{\text{Zone diameter}}$$

Determination of Indole Acetic Acid (IAA) and ammonia production: The amount of indole-3-acetic acid (IAA) produced by each PGPF isolate was determined by spectrometric method prescribed by Gordon and Weber (1951). The Absorbance value was taken at 550 nm for both samples and standards, from standard curve IAA was quantified. The selected fungal isolates were tested for the production of ammonia by adopting the method proposed by Cappucino and Sherman (2002).

Determination of antagonistic activity: The short listed better plant growth promoting fungal (PGPF) isolates were further screened and tested their bio-controlling ability against the selected plant pathogenic fungi (*Curvularia lunata, Fusarium oxysporum, Rhizoctonia solani* and *Trichosporium vesiculosum*) causing damping-off, root-rot, collar rot, stem rot and seedling wilt of many forestry species in nurseries. The pure culture of plant pathogenic fungi was brought from Forest Pathology Laboratory, Forest Protection Division, IFGTB, Coimbatore. Dual culture method described by Estrella *et al.* (2003) was adopted and determined the antagonistic efficacy of all the short listed microbial isolates under *in vitro*. The selected PGPF isolates were streaked on opposite sides of PDA plates and five day old fungal culture plug (6 mm) was kept at the centre of the plate, incubated at 25°C for 5 to 7 d. The PDA plates along with fungal culture without antagonistic microbes were kept as control. Percentage of inhibition by antagonistic microbes was recorded by measuring the reduction of fungal mycelial growth and compared it with control plate. Percentage of inhibition was calculated by using the following formula:

$$\text{Percent of inhibition} = \frac{C - T}{T} \times 100$$

Where, C is the mycelial growth of pathogenic fungi in control plate and T is the mycelial growth of pathogenic fungi in treatment.

Determination of siderophore production: Siderophore production was estimated quantitatively by spectrometric methods adopted from Schwyn and Neilands (1987) and Shin *et al.* (2001). The isolates were grown in MEB medium and processed as per the procedure and absorbance was read at 630 nm. Blank was maintained in entire process, isolates with <10% production considered as negative.

Qualitative screening of enzyme production: The fungal isolates were subjected to different enzymatic studies like chitinase, amylase, cellulase and protease activity for the identification of industrially potent fungi. The isolated fungal strains were screened for chitinase production on chitin-agar medium and confirmed by the method developed by Jenin *et al.* (2016). Production of proteolytic enzymes by fungal isolates was detected by using the Plate assay adopted from Hankin and Anagnostakis (1975). Production of cellulose enzyme by fungal isolates was detected using plate assay method developed by Lakshmi Narasimha Reddy *et al.* (2014). Production of amylase enzyme was detected by fungal isolates grown in Starch hydrolysis agar 7 d at 28°C. Formation of clear zone around the mycelium with the addition of gram's iodine confirmed the amylase production. The zone and the mycelium diameter were measured and the enzymatic index was calculated for all the enzymes by the following formula:

$$\text{Enzymatic Index} = \frac{\text{Diameter of zone}}{\text{Diameter of mycelium}}$$

Results and discussion

Physico-chemical analysis of soil samples: The soil samples collected under the root zone of all the three medicinal plants were processed and estimated soil nutrient parameters and data is presented in Table 1. It was observed that the soil samples of *A. indica* and *C. limon* showed acidic pH (5.51 and 5.87 respectively) and *A. marmelos* showed neutral pH (7.0). Electrical conductivity which represents total ion concentration was ranged from 0.03 to 0.13 dSm^{-1}. The bulk density was found to be same in all the samples. The available Nitrogen and Phosphorous was found to be in normal range and available potassium was observed in high in *A. marmelos* rhizosphere soil (536.2 kg ha^{-1}) when compared with other samples. Calcium and magnesium are very important elements for plant life and in the present study, the concentration of calcium and magnesium was found to be 1.8 to 28 Meq/100g Ca and 3.4 to 14.2 Meq/100g Mg respectively. The range of presence of

heavy metals such as iron, manganese, copper and zinc in all the samples was found to be in normal range when compared to standard permissible limit in all medicinal plant rhizosphere soil analyzed.

Estimation of population density of soil fungi: All the soil samples collected under the root zone of different medicinal plants were serially diluted and grown on PDA and RBA petri plates and population density of fungi was counted and calculated using Colony Forming Units (CFU). A total of 54 fungal isolates were obtained from three different rhizosphere soil samples. Among them, 22 pure isolates were obtained and maintained for further studies.

Estimation of soil spore population and percent root colonization of AM fungi: The roots and rhizosphere soil samples were also analyzed and estimated percent root colonization and soil spore population density of Arbuscular Mycorrhizal (AM) fungi and data is presented in Table 2. It was observed that all the rhizosphere soil samples had AM fungal spores but variation in population density such as 635, 589 and 482/100g soil in *A. marmelos, C. limon* and *A. indica* respectively. It is interesting to note that the AM fungal spores belong to three genera such as *Aculospora, Glomus* and *Scutellospora*, among them, the genus *Glomus* was found dominant. AM fungal root colonization was observed in all the root samples and maximum percent root colonization was recorded from the root samples of *A. marmelos* (86%) and this is followed by *C. limon* (79%). Less percent root colonization was recorded in root samples of *A. indica* (56%) during the period of investigation.

Determination of phosphate and zinc solublization efficacy: Experiment was conducted and screened the efficacy of phosphate and zinc solubilization efficacy of different fungal isolates isolated from the rhizosphere of three different medicinal plants under *in vitro* and data is presented in Figs. 1 and 2 respectively. Out of 22 fungal isolates screened, 17 fungal isolates had shown phosphate solubilization efficacy. Among those 17 isolates, seven isolates such as F2, F14, F15, F19, F20, F21 and F22 had shown >50% solubilization efficacy and they were short listed for further study. According to the results obtained from spectrometric analysis, the isolates such as F19 (A_{700}- 0.6707) and F15 (A_{700} -0.6496) solubilized phosphate at significant level than other isolates (Fig. 1). Similarly, all the fungal isolates were found to solubilize Zinc and represented by Zn solubilization index. Maximum amount of Zn solubilization was observed in F14 (1.93) isolate and F22 (1.93) isolate, followed by F15 (1.90) isolate (Fig. 2). The study is in accordance with the findings made by other earlier researchers on other host plants (Chang and Yang, 2009; Oniya *et al.*, 2015; Solaiman and Anawar, 2015; Mohan and Sangeetha, 2015; Mohan *et al.*, 2018).

Table 1. Physico-chemical analysis of soil samples.

Parameters	*Aegle marmelos*	*Azardirachta indica*	*Citrus limon*
pH	7.0	5.51	5.87
Electrical conductivity (ds/m)	0.13	0.05	0.03
Bulk density (gm/cc)	1.16	1.21	1.21
Organic carbon (%)	0.56	2.43	1.28
Available Nitrogen (kg/ha)	114.7	50.4	65.8
Available Potassium (kg/ha)	536.2	130.5	229.6
Available Phosphorus (kg/ha)	20.3	19.6	18.5
Calcium (Meq/100g)	28.8	2.2	1.8
Magnesium (Meq/100g)	14.2	3.4	4.4
DTPA-Cu (ppm)	0.5	1.3	1.6
DTPA-Zn (ppm)	0.11	0.2	0.3
DTPA-Mn (ppm)	8.1	1.1	1.6
DTPA-Fe (ppm)	0.8	5.6	6.0
Texture	Sandy loam	Loamy sand	Sandy loam

Table 2. Mean soil spore population and percent root colonization of AM fungi from three different medicinal plants.

Host plant	Number of spores/100 g of soil*	Percent root colonization*
Aegle marmelos	635	86
Azardirachta indica	482	56
Citrus limon	589	79
Host plant	Number of spores/100g of soil*	Percent root colonization*

Mean of 3 replications.

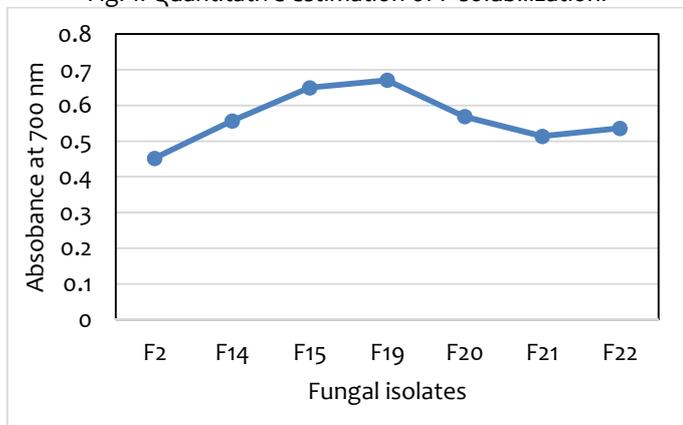Fig. 1. Quantitative estimation of P solubilization.

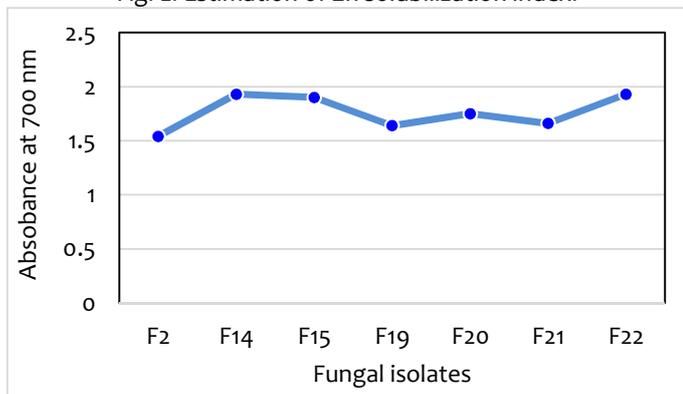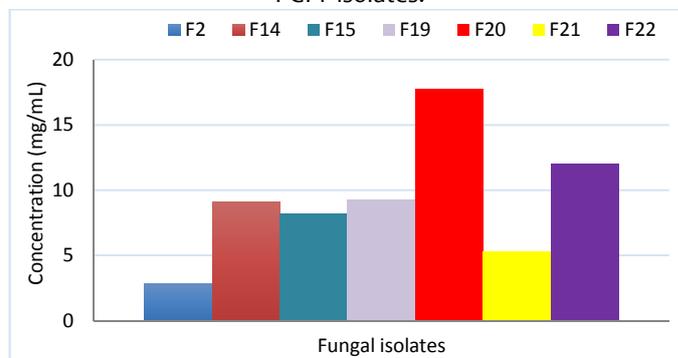Fig. 2. Estimation of Zn solubilization index.

Fig. 3. Determination of IAA production by selected PGPF isolates.

Chang and Yang (2009) and Oniya *et al.* (2015) have isolated "P" solubilizing fungi such as *Aspergillus niger* and *Penicillium* spp. from various rhizosphere soil samples and the solubilization indices of different isolates ranged from 1.06 to 2.29. Majority of microbes in the rhizosphere of different host plants can efficiently solubilize poorly soluble inorganic P and mineralize organic P sources (inaccessible to plants) and markedly increase plant growth in soils with low P availability (Solaiman and Anawar, 2015). Mohan and Sangeetha (2015) found that phosphate solubilising bacterial isolates showed a high rate of phosphate solubilization efficiency (SE) with *Bacillus megaterium* indicating a SE of 140% and *Bacillus subtilis*, a SE of 120%, making both the isolates as strong phosphate solubilizers.

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Fig. 4. Antagonistic potential against the selected plant pathogenic fungi.
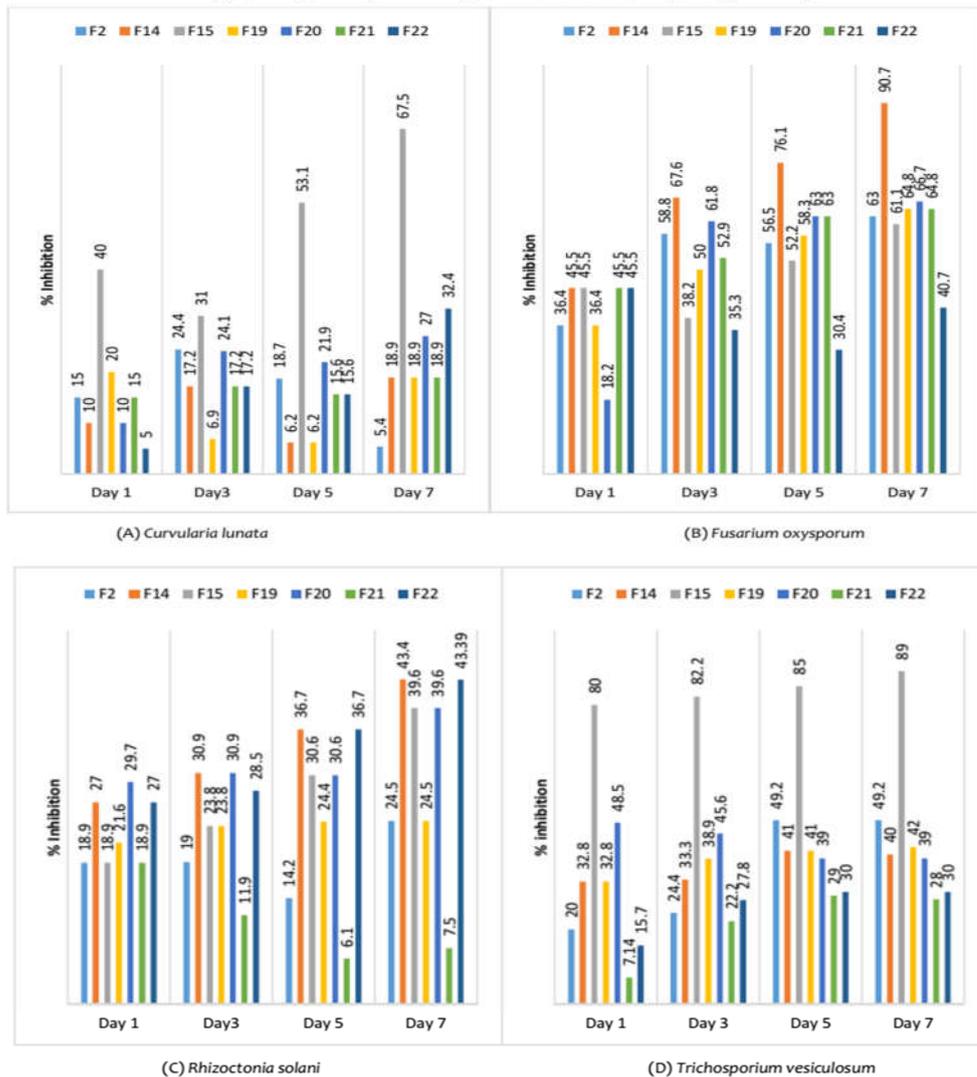
Mohan *et al*. (2018) recorded that two each of bacterial and Actinomycete isolates revealed 75% and 80% respectively and one fungal isolate revealed 100% phosphate solubilisation potential as compared to other isolates obtained from the rhizosphere of *Gmelina arborea* plants. Fungal strains isolated from sugarcane and sugar beet rhizosphere showed SI in range of 1.13 to 1.59 (Mahamuni *et al.*, 2012).The zinc plays a main role in nutrient supplement for plants. Since inorganic zinc compounds are commonly found in soil as insoluble form. Soil fungi have important influence in bio-geochemical cycle and are involved in solubilization of insoluble metal compounds such as Zinc. In this study, maximum amount of Zn solubilization was noticed in F14 (1.93) and F22 (1.93) followed by F15 (1.90) as similar work was done by Sutjaritvorakul *et al.* (2011) and their isolate showed 86.95% solubilization.

Determination of IAA production: Indole Acetic Acid (IAA) production is a major property of rhizosphere fungi that stimulate and facilitate plant growth. In this study, the selected PGPF fungal isolates were screened for IAA production and data is presented in Fig. 3. It was recorded that the isolate F20 produced maximum IAA (17.78 mg/mL) as compared to other isolates and the fungal isolate F2 produced the least (2 mg/mL) amount of IAA. All the fungal isolates except F2 isolate showed ammonia production. There are many earlier reports revealed the production ability of phytohormones by different microbes *viz.,* *Azotobacter* (Wendo *et al.,* 2002), *Azospirillum* (Yasmin *et al.,* 2007) and also Rhizobial bacteria (Arshad and Frankenberger, 1998). Similar kind of experiment was done by Mohan *et al.* (2015) to estimate IAA production by different ectomycorrhizal (ECM) fungi under *in vitro* condition and it was observed that the highest IAA produced by ECM fungi *L. fraterna* (28.18 µg/mL), *P. albus* (25.32 µg/mL) and *S. citrinum* (23.40 µg/mL).

Determination of antagonistic potential of selected PGPF:
Experiment was conducted and screened the antagonistic efficacy of all the short listed PGPF isolates against the selected plant pathogenic fungi *viz.,* *C. lunata*, *F. oxysporum*, *R. solani* and *T. vesiculosum* and the results are presented in Fig. 4. It was observed that the fungal isolate F14 showed maximum inhibition rate of 90.7% and least by F22 (40.7%) against *F. oxysporum*. It was recorded that the fungal isolate F15 showed maximum inhibition rate of 89% and the least inhibitory effect was noticed in F21 (28%) against *T. vesiculosum*. Similarly, F15 showed maximum inhibition of 67.5% and least inhibition rate was shown by F2 (5.4%) against *C. lunata*. Among different pathogens tested, maximum percent inhibition was recorded on *F. oxysporum* growth as compared to the control plate which may be caused due to the presence of volatile compounds produced by the fungal isolates. Similar work was done by Mohan *et al.* (2015), according to them antagonistic activity of ECM fungus *S. subluteus* against plant pathogenic fungi, *F. oxysporum* was 58.21%. This study shows that the fungal isolate F14 showed better growth inhibition of different pathogenic fungi than the ECM fungi. Meena *et al.* (2017) found that better growth inhibition of *Fusarium oxysporum* may be caused due to the presence of volatile compounds produced by the fungal isolate.

Determination of Siderophore production:
Another experiment was conducted and determined the ability of siderophore production by different PGPF isolates and data is presented in Fig. 5. It was observed that the fungal isolate F15 recorded the maximum amount (30.31%) of siderophore production and this is followed by isolate F22 (20.1%). The fungal isolate F19 recorded least amount (11.11%) of siderophore production. The findings of the study are in concurrence with the findings made by other researchers (Ghosh *et al.,* 2015) and they recorded that *P. aeroginosa*-1 produced maximum of 80.50% siderophore.

Screening of enzyme production: Fungi are generally regarded as safe strains as they produce extracellular enzymes, which are easier to be recovered from fermentation broth. In the present study, all the fungal isolates were screened qualitatively for the Chitinase, Amylase, Protease and Cellulase production and the data are given in Fig. 6. It was observed that the isolate F21 revealed maximum enzyme production as compared to other fungal isolates. Among different enzymes, Protease was found more and this is followed by cellulose enzyme.

Conclusion
The fungal isolates F14 and F15 were considered as potent bio-inoculants through all the screening tests done. They were characterized and sequenced at molecular level and identified as *Penicillium citrinum* and *Aspergillus oryzae*.

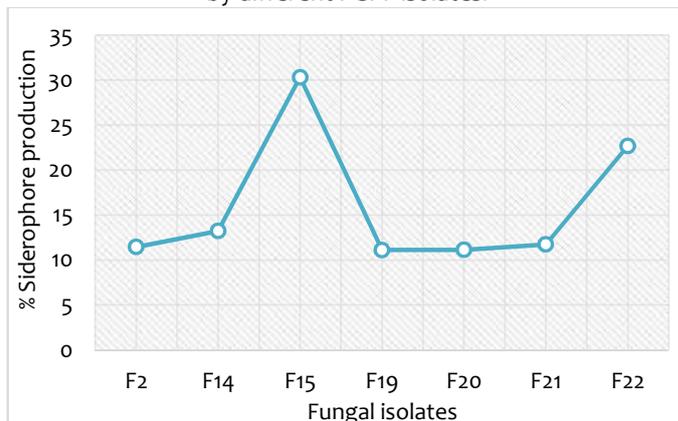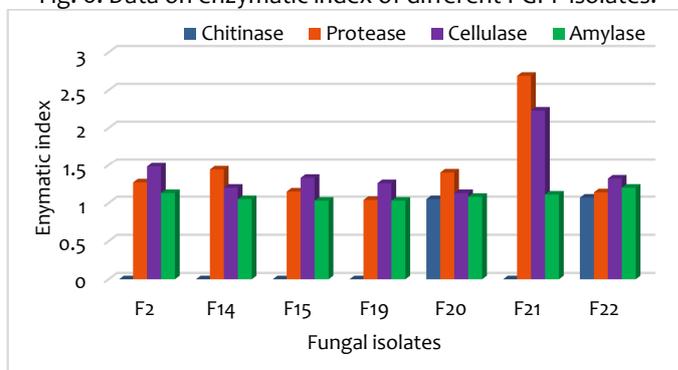Fig. 5. Determination of percentage siderophore production by different PGPF isolates.

Fig. 6. Data on enzymatic index of different PGPF isolates.

The characterized rhizosphere soil fungi have phosphate and zinc solubilizing ability; IAA production and antagonistic efficacy against different plant pathogenic fungi. These potential PGPF can be brought out in the form of bio-control product which improves the plant growth by suppressing different soil and root borne pathogens in nursery.

Acknowledgements

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