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Diversity of Arbuscular Mycorrhizal Fungi (AMF) in the Rhizosphere of *Helianthus annuus* L.

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**Abstract:** In this investigation an attempt was made to reveal the root colonization and species diversity of AMF in the Rhizosphere of sunflower, collected from different areas which differed in physico-chemical parameter. The higher colonization was observed at Arasadiptatti (Pudukkottai), Thalaimalaipatti (Trichy) Thirukkanurpatti (Thanjavur) and the lower colonization was observed at Thirumalaisamuthirum (Thanjavur). The colonization among root samples collected from ten different soils, ranged from 20%-70%, this may due to some biotic and abiotic stress. Altogether fourteen species of AMF were isolated, of which seven species were contributed by *Glomus* spp., three by *Gigaspora* spp. and two by *Acalospora* spp. and *Scutilospora* spp., The spore densities of soils studied varied from 210 to 740/100g soils. The highest isolation frequency, of 60% was represented by *Glomus mossae* and the lowest was by *Glomus* species 1, *Gigaspora* sp1, *Gigaspora* sp2. Among the ten soils, species richness and Shannon-Wiener index of diversity ($H'$) were found to be greater at Vallamputhur (Thanjavur), Sampson’s index of dominance (D) was found to be high at Kummankulum (Pudukkottai).

**Key words:** AMF diversity - Colonization - Spore density - Sunflower

**INTRODUCTION**

Arbuscular Mycorrhizal (AM) fungi are the symbiotic fungi, which form mutualistic association with feeder roots of most terrestrial plants. These fungi are the major component of soil microbial communities in terrestrial eco systems, which can form mutually beneficial symbiosis with 90% of vascular plants [1]. On global basis, mycorrhizae occurs in 83% of dicots and 79% of monocots, where as all gymnosperms are mycorrhizal. Dense AM fungi infection is common in most species of Leguminosae and Gramineae. Most of the economically important crops are infected by AM fungi [2]. These fungi enter cortex of roots to obtain carbon from their host plants, while assisting the plants with the uptake of phosphorus and the other mineral nutrients from soil [3]. This association is beneficial to plants because, phosphorus is a major essential element for growth and development. It has been observed that alkaline and acid phosphatase enzyme activities of the rhizosphere soil were much related to AM fungal activity [4]. The other functions attributed to AM fungi include production of plant growth hormones, protection of host roots from pathogens, uptake of heavy metals, salinity tolerance [5]. Distribution, diversity, abundance and functioning of AM fungi are primarily based upon the root colonization and spore count which further depend upon many environmental factors [6]. In addition to the sensitivity to soil type, some other factors that affect behavior of AM fungi are host plant, crop rotation; soil pH, moisture content of soil, soil temperature, nutrient levels and interaction with other soil biota. The objective of this investigation is to reveal the species diversity of AM fungi in Rhizosphere of sunflower in different agroecological stations.
**MATERIALS AND METHODS**

**Location of Sampling Sites:** The study sites were located in southern districts of Tamil Nadu in South India, which included Pudukkottai (Arasadiptti, Kummanikulam, Manjanviduthi), Thanjavur (Thirukkanur, Vallamputhur, Thirumalaismuthiram), Trichirappalli districts (Meickalnaickanpatti, Valavanthi, Thalaimalaiapatti, M.Kalathur). Location the study sites are shown in Fig. 1. Tiruchirappalli is located at 10.8050°N 78.6856°E. The average elevation is 88 metres (289 ft). It is located almost at the geographic centre of the state of Tamil Nadu. Tiruchirappalli experiences a moderate climate from August to October, tempered by heavy rain and thundershowers and cool and balmy climate from November to February. Fog and dew are rare and occur only during the winter season. Pudukkottai district covers an area of 4663 Sq. Km. which has a coast line of 39 Kms. The district is located between 78.25' and 79.15' of the East Longitude and between 9.50' and 10.40' of the North Latitude. Thanjavur is situated beside the mighty River Cauveri, Thanjavur is geographically located in between 10.8°N and 79.15°E in the South Indian state of Tamil Nadu. Thanjavur has a tropical climatic condition. During summers the average temperature of the city rises to 36.6°C, while during the winter season, the average temperature goes down to 22°C. The city of Thanjavur experiences heavy rain of about 111.37 cm during the rainy season.

**Collection of Root and Soil Samples:** Extensive field survey was carried out in order to collect the root and rhizospheric soils samples of sunflower crop plants from different agro ecological zones of Pudukkottai, Thanjaur and Trichirappalli districts of Tamil Nadu during the year 2011 -2012. Samples were collected randomly. Rhizospheric soils at a depth of 4-16 cm from 5 different locations in each study site were collected in sterile polyethylene bags using soil auger. Approximately 500gm of rhizosphere soil was collected. Soil samples were air-dried and stored at 4°C for processing. A portion of the soil samples was analyzed for soil physio-chemical parameters like pH, electrical conductivity, available phosphorus and available potassium. Remaining soil samples of 200g was used to isolate AM fungal spores. The root samples were washed thoroughly with running tap water to remove the adhered soil particles. Then roots were cut into small pieces of about 1cm and used for the assessment of percentage colonization of AMF.

**Estimation of Root Colonization:** The technique of Phillips and Hayman [7] modified by Koske and Gemma [8] was used throughout the research work for determining the AM infection in sunflower roots growing under field condition. This procedure include various steps i.e. depigmentation of roots by 10%KOH, washing with tap water, acidification of roots with 2N HCl, staining root with 1% trypsin blue. Segments of 0.5 cm of were
Number of AM positive segments
Root colonization (%) = \( \frac{\text{Number of AM positive segments}}{\text{Total number of segments observed}} \times 100 \)

**Isolation of AM Fungi:** AM spores were extracted from the collected soil samples by wet sieving and decanting method to obtain viable and debris free AM spores [9]. Soil mass 100gm was suspended in 500ml of distilled water, heavier particles gradually settle down the bottom of the container. The suspension was passed through the series of sieves of the following pores dimensions 710mm, 425mm, 250mm and 45mm. Residues from the last two sieves were washed and collected in the beaker. After 5 minutes the supernatant was filtered through Whatmann No. 1 filter paper. The filter paper containing the residues was placed on the Petri plates and was observed under light binocular microscope for spore counting. The healthy and fresh spores were isolated with the help of needle and were placed on the slide and were mounted in Poly Vinyl Lacto Glycerol (PVLG) to make permanent slides.

**Identification of AM Fungi:** The isolated AM spores were microscopically examined for spore morphology viz. size of spores, nature and number of spore walls, spore inclusions and hypha attachment. The isolated AM spores were then identified on morphological basis by referring to the latest taxonomy on AM fungi given by Schenck and Perex [10].

**Result and Discussion**

**Diversity of AM Fungal Spores and Colonization:** Rhizosphere soil samples collected from various localities revealed the presence of several species of AM fungi. A total of 4,446 spores of AMF were wet sieved from soil sample collected from ten different agro ecosystems. From which 14 species were identified. The identified species were *Glomus mossae, G. fasciculatum, G. microsporum, G. aggregatum, G. intradices, Glomus sp1, Glomus sp2, Gigaspora sp1, G. gigantea, Gigaspora sp2, Acalospora scrobiculata, A. lavis, Scutulospora sp1, Scutulospora sp2* (Table 3).

<table>
<thead>
<tr>
<th>Table 1: Diversity measures used to describe AM communities</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spore density (SD)</strong></td>
</tr>
<tr>
<td><strong>Species richness (SR)</strong></td>
</tr>
<tr>
<td><strong>Relative abundance (RA)</strong></td>
</tr>
<tr>
<td><strong>IF (Isolation Frequency)</strong></td>
</tr>
<tr>
<td><strong>Simpson’s index of dominance</strong></td>
</tr>
<tr>
<td><strong>Shannon–Wiener index of Diversity (H’)</strong></td>
</tr>
</tbody>
</table>

\( \text{P}_i = \frac{n_i}{N} \), where \( n_i \) is the spore numbers of a species and \( N \) is the total number of identified species per sampling sites.
Table 2: Relative abundance (RA) and Isolation frequency of AM in Rhizosphere soil of Sunflower

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of the Organisms</th>
<th>Relative Abundance (RA)%</th>
<th>Isolation Frequency (IF) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glomus mossae</td>
<td>16.17</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>Glomus fasciculatum</td>
<td>21.64</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>Glomus microsporum</td>
<td>7.21</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Glomus aggregatum</td>
<td>9.68</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>Glomus intradices</td>
<td>6.52</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>Glomus sp1</td>
<td>0.68</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>Glomus sp2</td>
<td>2.40</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>Gigaspora sp1</td>
<td>0.68</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>Gigaspora gigantea</td>
<td>4.12</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>Gigaspora sp2</td>
<td>1.37</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>Acalospora scrobiculata</td>
<td>8.59</td>
<td>40</td>
</tr>
<tr>
<td>12</td>
<td>Acalospora lavis</td>
<td>13.40</td>
<td>50</td>
</tr>
<tr>
<td>13</td>
<td>Scutilospora sp1</td>
<td>4.12</td>
<td>30</td>
</tr>
<tr>
<td>14</td>
<td>Scutilospora sp2</td>
<td>4.46</td>
<td>40</td>
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</tbody>
</table>

Table 3: Diversity measurement of AMF community

<table>
<thead>
<tr>
<th>Factors</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S8</th>
<th>S9</th>
<th>S10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore density (SD)/100g of soil</td>
<td>704</td>
<td>470</td>
<td>350</td>
<td>284</td>
<td>210</td>
<td>413</td>
<td>425</td>
<td>530</td>
<td>680</td>
<td>560</td>
</tr>
<tr>
<td>Species Richness (SR)</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Shannon-Wiener index of diversity (H’)</td>
<td>1.6068</td>
<td>1.52</td>
<td>1.1372</td>
<td>0.5297</td>
<td>1.1658</td>
<td>0.7355</td>
<td>0.9189</td>
<td>1.0862</td>
<td>1.805</td>
<td>0.9794</td>
</tr>
<tr>
<td>Sampson’s index of dominance (D)</td>
<td>0.2291</td>
<td>0.2254</td>
<td>0.3291</td>
<td>0.1498</td>
<td>0.2043</td>
<td>0.5803</td>
<td>0.4616</td>
<td>0.1952</td>
<td>0.1665</td>
<td>0.4618</td>
</tr>
</tbody>
</table>

Meickalnaickanpatti-S1, Valavanthi -S2, Thalaimalaiapatti -S3, M.Kalathur -S4, Arasadipatti- S5, Kummankulum-S6, Manjanviduthi -S7, Thirukkanupatti -S8, Vallamputhur –S9, Thirumalaisamuthirum- S10

Supriya Gaur and Purshotam Kaushik [14] isolated a total of 16 species of AM from three medicinal plants of which more than fifty percent of the total species identified belonged to the genus *Glomus*. Like wise Sharma et al. [15] described seven species of *Glomus* from sunflower rhizosphere. Similarly in this study also seven species of *Glomus* were isolated and identified. Sharma et al. [16] identified seven species of *Acalospora* from Sunflower rhizosphere soil of Haryana, India. Whereas, in the present study two *Acalospora* species (*Acalospora lavis* and *A. tuberculata*) have been identified (Table 2). In this investigation the species richness was maximum at Vallamputhur (SR 7%, SD 680/100g soil) however the spore density was minimum when compared to Meickalnaickanpatti (SR 5%, SD 704/100g soil). Spore density and species richness were found to be less in M. Kalathur (SR 2%, SD 284/100g soil). Kummankulum, Manjanviduthi and Thirumalaisamuthirum had the same SR (3%) value but differed in SD (413, 425, 560) respectively; similar results were recorded at Thalaimalaiapatti and Arasadipatti (Table 3). Correlation analysis demonstrated that spore density of AMF species was positively correlated with species richness (SPSS software version16.0, r = 618) (Fig. 2). The result of the present study coincides with the previous reports [17, 18]. Based on relative abundance and isolation frequency, it was observed that *Glomus fasciculatum* was most dominant (21.64% of RA) followed by *Glomus mossae* (16.17% of RA) and *Acalospora lavis* (13.40% of RA). However *Glomus mossae* contributed to greater isolation frequency (60%) and was widely distributed, followed by *Glomus fasciculatum* and *Acalospora lavis* (50%). This finding was in accordance Karthikeyan and selvaraj [19]. Correlation analysis revealed that there was positive correlation between relative abundance and isolation frequency (SPSS software version16.0, r = 815) (Fig. 3). This finding was supported by earlier works [20]. They suggested that AMF colonization varied widely among different vegetable crops and fruit yielding crops, *Glomus* was represented by10 species. Furthermore, Shannon-Wiener index of diversity (H’) and Sampson’s index of dominance (D) showed greater diversity (Table 3).

**AM Fungal Colonization in Root of Sunflower:** Arbuscular mycorrhizal fungal taxa have a specific multidimensional niche determined by the plant species that are present at the site and by edaphic factors such as pH, moisture content and phosphorus (P) and nitrogen (N) availability [21]. Variation in spore density and percent
Fig. 2: Correlation between spore density and species richness

Fig. 3: Correlation between Isolation Frequency (IF) and Relative Abundance (RA)

Table 4: Percentage root colonization of AMF in roots of Sunflower

<table>
<thead>
<tr>
<th>S.No</th>
<th>Study sites</th>
<th>Percentage of AM Colonization in Root samples</th>
<th>No. of vesicles per Root samples</th>
<th>No. of Arbuscles per Root samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S1</td>
<td>50</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>S2</td>
<td>60</td>
<td>3</td>
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<td>3</td>
<td>S3</td>
<td>70</td>
<td>5</td>
<td>4</td>
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<tr>
<td>4</td>
<td>S4</td>
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<td>6</td>
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<td>5</td>
<td>S5</td>
<td>70</td>
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<td>5</td>
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<td>S8</td>
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</tr>
<tr>
<td>10</td>
<td>S10</td>
<td>30</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

colonization among different sampling sites attributed to host specificity, edaphic and climatic conditions [22]. Table 4 reveals that the percentage of AMF colonization of sunflower root varied greatly among different study sites. Highest infection rate of 70% was observed at Thalaimalaiappatti, Arasapidappatti, Thirukanurappatti and lowest infection rate of 30% was recorded at Thirumalaisamuthirum. The colonization rate of 35% in sunflower root collected from marginal soil and of 29% sample collected from fertile soil was observed [23]. The results obtained in this study coincides with the previous reports [24], who examined the seasonal dynamics of several soil variables (soil pH, temperature, moisture) with specific interest to determine the rate of arbuscular mycorrhizal root colonization, vesicles and arbuscles formation in the root and AM fungal spore population in the rhizosphere of five medicinal plants and reported that the season and edaphic conditions are crucial for the development of AM fungi.

REFERENCES


ABSTRACT
The main aim of this investigation was to determine the antagonistic activity of *Tricoderma* spp isolated from rhizosphere soil of sunflower. Two species namely *Trichoderma viride* and *Trichoderma koningii* were isolated. In dual culture method *Trichoderma viride* showed maximum growth inhibition of 72.20% against *Fusarium oxysporum* whereas, *Trichoderma koningii* effectively inhibited *Pythium debarianum* (57.42%). The result of volatile assay revealed *Pythium debarianum* was effectively inhibited by both antagonists and in non volatile assay *Pythium debarianum* was greatly inhibited followed by *Fusarium oxysporum*, *Fusarium javanicum*, *Macrophomina phaseolina*. The *Trichoderma viride* and *Trichoderma koningii* were recorded for their maximum cellulolytic and chitinolytic activity (3.6U/ml and 0.37IU/ml, 2.75 IU/ml and 0.29IU/ml), respectively.

Keywords: *Tricoderma* spp, phytopathogens, biocontrol, chitinase, cellulase.

INTRODUCTION
Fungal Phytopathogens are the cause of many plant diseases and much loss of crop yields, especially in subtropical and tropical regions [1]. *Macrophomina phaseolina* (Tassi) Goid, a soil borne fungus causes charcoal rot over 500 plant species and has a wide geographic distribution [2]. Chemical fungicides are extensively used in contemporary agriculture. However these products may cause problems such as environmental pollution and have adverse effects on human health. Microorganisms as bio control agents have high potential to control plant pathogens and have no negative effect on the environment (or) other non target organisms. *Tricoderma* spp are used as effective biocontrol agents against several soil borne fungal plant pathogens including *Macrophomina phaseolina*, *Fusarium* spp, *pythium* spp [3]. The bio control exercised by *Tricoderma* can occur by several antagonistic mechanisms such as nutrient competition, antibiotic production and mycoparasitism. Mycoparasitism has been reported as the major antagonistic mechanism displayed by *Tricoderma* spp. After host recognition, *Tricoderma* spp attaches to the host hyphae via coiling and penetrate the cell wall by secreting cell wall degrading enzymes which allow them to bore holes into the fungal host and extracts nutrients for their own growth. Most phytopathogenic fungi have cell wall that contain chitin as a structural backbone arranged in a regularly ordered layers and β, 1-3 glucan as a filling material arranged in an amorphic manner. Chitinases and β, 1-3 glucanases have been directly involved the mycoparasitism interaction between *Tricoderma* spp and its host [4].

MATERIALS AND METHODS
Isolation of antagonist
The fungal antagonists were isolated from the rhizosphere soil of sunflower; using serial dilution and pour plate technique on potato dextrose agar medium (PDA). The antagonist were purified and identified based on morphological and micropscopical characters. The isolates were maintained on PDA slants at 4°C through out the study.

Isolation of phytopathogens
Diseased plant tissues were washed under running tap water to remove surface soil and other contaminant. Infected tissues were cut into small pieces and placed in 1% sodium hypochloride for five minutes, placed on PDA plates and incubated at 28°C. The cultures were purified by hyphal tip method [5] and maintained on PDA slants by storing it under refrigeration (4°C). Phytopathogens isolated from infected tissues were identified based on morphological and microscopical charters.

Growth inhibition assay by dual culture method
Interaction between antagonistic fungi and pathogenic fungi were determined by the method of Dennis and Webster [6]. A 5 mm diameter mycelial disc from the margin of the *Trichoderma* one week-old culture and the pathogens *M. phaseolina*, *Fusarium javanicum*, *F. oxysporum*, *Pythium debarianum* were placed on the opposite side of the plate at equal distance from the periphery. In control plates (without *Trichoderma*), a sterile agar disc was placed at opposite side of the pathogen *M. phaseolina* inoculated disc. The plates were incubated at 28 ± 2°C and observed after 7 days.

Assay for volatile metabolites of *Tricoderma* spp
Productions of volatile metabolites by *Trichoderma* spp were assayed as described by Dennis and Webster [6] and Goyal et al.,[7] with slight modifications. The *Trichoderma* isolates were centrally inoculated by placing 3mm disc taken from three days old cultures on the PDA plates and incubated at 28 ± 2°C for three days.
The top of each petridish was replaced with bottom of PDA plate inoculated centrally with the pathogen. Petridish with PDA medium without *Trichoderma* spp at the lower lid and the upper lid with pathogens was maintained as control. The pair of each petridishes were sealed together with paraffin tape and incubated for 4-6 days. After incubation the inhibition of mycelial growth was calculated.

**Assay for non volatile metabolites of *Trichoderma* spp**

The effects of non volatile metabolites produced by the *Trichoderma* spp were determined by following the methods of Dennis and Webster [6]. The isolates of *Trichoderma* spp were grown on minimal synthetic medium (MSM) containing the following components (in grams per liter): MgSO₄.7H₂O, 0.2; K₂HPO₄, 0.9; KCl, 0.2; NH₄NO₃, 1.0; FeSO₄.7H₂O, 0.002; MnSO₄, 0.002 and ZnSO₄, 0.002. The medium was supplemented with the appropriate carbon source for cellulose and chinase assay (commercially available Carboxy methyl cellulose, Chitin). The pH was set to 6.3 with 50mM phosphate buffer (commercially available Carboxy methyl cellulose, Chitin). The pH was set to 6.3 with 50mM phosphate buffer (0.05 M, pH 5.2) and 1 ml distilled water. The mixture was then incubated in shaking water bath at 50°C for 10 min, thereafter 3 ml of 3; 5-dinitrosalicylic acid reagent was added. The mixture was then placed in a boiling water bath for 5 min, after cooling, the developed color, as indication to the quantity of released N-acetylglucosamine (NAGA), was measured spectrophotometrically at 575 nm. The amount of NAGA was calculated from standard curve of NAGA.

**RESULTS AND DISCUSSIONS**

**Table-1.** Percent of inhibition by *Trichoderma* isolates after 7 days of inoculation in dual culture.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Antagonists</th>
<th>T. viride</th>
<th>T. koningi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pythium debarianum</td>
<td>66.24</td>
<td>57.42</td>
<td></td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>72.20</td>
<td>52.75</td>
<td></td>
</tr>
<tr>
<td>Fusarium javanicum</td>
<td>64.43</td>
<td>44.54</td>
<td></td>
</tr>
<tr>
<td>Macrophomina phaseolina</td>
<td>42.36</td>
<td>47.24</td>
<td></td>
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**Table-2.** Percent of inhibition by *Trichoderma* isolates in volatile assay method.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Antagonists</th>
<th>T. viride</th>
<th>T. koningi</th>
</tr>
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<td>Pythium debarianum</td>
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<td>60.17</td>
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<tr>
<td>Fusarium oxysporum</td>
<td>60.28</td>
<td>54.20</td>
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<tr>
<td>Fusarium javanicum</td>
<td>68.2</td>
<td>59.4</td>
<td></td>
</tr>
<tr>
<td>Macrophomina phaseolina</td>
<td>49.10</td>
<td>40.9</td>
<td></td>
</tr>
</tbody>
</table>

**Table-3.** Percent of inhibition by *Trichoderma* isolates in non volatile (culture filtrates) assay method.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Antagonists</th>
<th>T. viride</th>
<th>T. koningi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pythium debarianum</td>
<td>76.66</td>
<td>60.21</td>
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<tr>
<td>Fusarium javanicum</td>
<td>66.76</td>
<td>54.43</td>
<td></td>
</tr>
<tr>
<td>Macrophomina phaseolina</td>
<td>39.67</td>
<td>37.67</td>
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</tbody>
</table>
which play an important role in controlling the plant pathogens [15]. The study non volatile assay revealed that both the antagonist effectively inhibited the mycelial growth of phytopathogen in the following order Pythium debarianum > Fusarium javanicum >, Fusarium oxysporum >, Macrophomina phaseolina. The volatile and non volatile copound from Trichoderma effectively inhibited the growth of Colletotrichum capsici [16]. Muthukumar et al.,[17] and Christy Jeyaseelan et al.,[18] recorded maximum growth inhibition of T. viride against Pythium aphanidermatum through more production of volatile and non volatile compounds.

Assay for cell wall degrading enzyme activity

Chitinolytic and glucanolytic (fungal cell wall-degrading enzymes) have been suggested to be the primary determinants of biocontrol by Trichoderma spp [19]. The highest cellulase activity of the isolate of T. viride and T. koningii observed were 3.15IU/ml and 2.75 IU/ml, respectively after 72 hours incubation. Vinith Kumar Mishra, [20] observed that the T. viride exhibited highest cellulase activity of 3.6U/ml and it was found to be suppress the mycelial growth of Pythium aphanidermatum. In this investigation the maximum chitinase enzyme was 0.37IU/ml in the case of T. viride, whereas T. koningii exhibited 0.29IU/ml activity. Eman Faith Sharaf et al.,[21] reported the maximum production of chitinase enzyme (23.8U/ml) by a most potent T. viride isolate using colloidal chitin prepared from Shrimp shell waste as sole carbon source. Sevugaperumal Ganesan and Rajagobal Sekar [22] explored the antagonistic activity of nine species of Trichoderma against Rhizoctonia solani, and reported that the chitinolytic activity of the antagonists ranged from 5.51IU/ml to 0.12 IU/ml. T. harzianum culture filtrates, possessing chitinase and glucanase activities, were capable of hydrolyzing dried or fresh mycelium of the phytopathogenic fungus Sclerotium rolfsii. Growth of Sclerotium rolfsii was significantly inhibited by the enzyme preparations from T. harzianum [23]. Agrawal and Kotasthane [24] evaluated the production of chitinase enzyme by Trichoderma spp using colloidal chitin which was derived from the cell wall of plant pathogen Rhizoctonia and commercial chitin.

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SCREENING OF RHIZOBACTERIA FOR PLANT GROWTH PROMOTING TRAITS AND ANTFUNGAL ACTIVITY AGAINST CHARCOAL ROT PATHOGEN MACROPHOMINA PHASEOLINA

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ABSTRACT

Plant growth promoting rhizobacteria stimulate plant growth and suppress the colonization of phytopathogens by involving direct and indirect mechanisms. In our investigation ten predominant rhizobacterial isolates belonging to six genera were isolated and characterized for their multiple growth promoting attitudes and antagonistic activity. The results revealed that 70% of the isolates produced phytohormone IAA, 80% of isolates produced ammonia and HCN. Among the ten isolates, four (40%) exhibited phosphate solubilizing and siderophore producing ability. The cell wall degrading enzymes such as cellulase, protease, chitinase activities of isolates were also recorded. They were in the range of 50%, 10% and 60 %, respectively. The antifungal activity of the isolates were tested against Macrophomina phaseolina, among the ten bacterial isolates four (40%) effectively inhibited mycelial growth of plant pathogen. The bacterial strain which showed better results for all the test was identified as Bacillus amylo liquefacience by 16SrRNA sequencing.

KEY WORDS: Rhizobacteria, IAA, Siderophores, HCN, Cell wall degrading enzymes, Macrophomina phaseolina.

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B - 177
INTRODUCTION

Sunflower is mainly grown in Rabi season as a rain fed crop mostly by marginal farmers. Charcoal rot, is a major potential threat for crops such as sunflower and soybean. Charcoal rot epidemics are common under severe conditions such as water scarcity and other biotic and abiotic stresses. The pathogen *Macrophomina phaseolina* (Tassi) Goid is an anamorphic ascomycete of the family Botryosphaeriaceae and causes the disease charcoal rot on a broad range of plants in many areas of the world. Its infection on sunflower was first reported from India in 1973. The fungus is reported to be soil, seed and stubble borne. The evidence suggests that it is primarily a root inhibiting fungus and produces a tuber or cushion shaped 1–8 mm diameter black sclerotia. These sclerotia serve as a primary means of survival. Some chemical fungicides are used to control the charcoal rot pathogen. However these chemicals are not ecofriendly, hence an alternative method is needed to manage this disease. Soil-borne diseases have been controlled more recently by means of certain beneficial bacteria that are indigenous to the rhizosphere of plants. The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large and metabolically active groups of bacteria known as plant growth promoting rhizobacteria (PGPR). PGPR rapidly colonize the rhizosphere and suppress the pathogenic microorganisms at the root surface. PGPR use one (or) more direct mechanism of action to improve plant growth and health. Improvement of plant uptake by phosphate solubilization (or) nitrogen fixation and phytohormone production like indole-3-acetic acid are examples of mechanism of direct influence on plant growth. Biological control of plant pathogens and deleterious microbes, through production of antibiotics, lytic enzymes, hydrogen cyanide and siderophore (or) through competition for nutrient and space can significantly improve plant health and promote growth by increasing of seedling emergence, and yield. Recent studies have demonstrated the importance of strains of non-pathogenic PGPR in enhancing plant resistance. Therefore an investigation was carried out to reveal the plant growth promoting activities such as IAA production, Ammonia production, HCN production, Phosphate solubilization, siderophore production, cell wall degrading enzyme activities and antifungal activity against *Macrophomina phaseolina* of rhizobacterial isolates.

MATERIALS AND METHODS

**Isolation of Bacteria from Rhizosphere soil**

The Rhizosphere soil samples of sunflower plant were collected in polyethylene bags from Pudukottai districts, Tamil Nadu and transported to the laboratory and kept in refrigerator (4°C) for further process. The Rhizobacteria were isolated on nutrient agar medium and they were purified by streaking on the same medium. The purified colonies were maintained as pure culture with periodic transfer to fresh media and stocked for further use. All the isolates were identified at genus level based on colony morphology, Gram’s staining, motility test and biochemical characteristics.

**Production of Indole Acetic Acid (IAA)**

Indole acetic acid production was detected as described by Bric et al.. Bacterial isolates were inoculated in nutrient agar amended with L-Tryptophen and incubated at 37°C for 48hrs. Fully grown cultures were centrifuged at 3000rpm for 30 minutes, the supernatant (2ml) was mixed with two drops of orthophosphoric acid and 4ml of the Salkowski reagent (50 ml 35% of perchloric acid, 1ml of 0.5mFeCl₃ solution). Development of pink colour was indicative of IAA production.

**Ability for Phosphates solubilization**

Phosphate solubilizing ability of the isolate was checked on Pikovskaya (PVK) medium, incorporated with tricalcium phosphate (Ca₃(PO₄)₂). The isolates were spot inoculated on PVK medium. Formation of transparent halo
zone around the developing colonies indicated phosphate solublizing ability of the isolates.

**Assay for NH3 production**

The rhizobacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated into 10 ml peptone water and incubated at 30°C for 48 hrs. Nessler’s reagent (0.5 ml) was added to each tube. Development of brown to yellow colour was a positive test for ammonia production.

**Hydrogen Cyanide production (HCN)**

Production of HCN was determined using the modified procedure of Millar and Higgins. All the bacterial strains were grown on Trypticase Soy Agar (TSA) plates. Sterilized Whatman No. 1 filter paper strips were soaked in picric acid solution (2.5 gm of picric acid, 12.5gm of Na₂CO₃, in 1000ml of distilled water) and were placed in the lid of each Petri dish. Dishes were sealed with parafilm and incubated at 28°C for 48 hrs. A change in colour of the filter paper strip from yellow to light brown, brown or reddish brown was recorded as an indication of weak, moderate or strong production of HCN by each strain, respectively.

**Screening for siderophore production**

Production of siderophores by bacterial strains were performed by plate assay according to Schwyn and Neillands. The tertiary complex, Chrome azurall S (CAS) / Fe³⁺ / hexadecyl trimethyl ammonium bromide served as an indicator. Forty eight hour old culture of the strains was streaked onto the Succinate medium amended with indicator dye. The formation of bright zone with yellowish fluorescent colour by the culture in the dark colored medium indicated siderophore production. The result was scored either positive or negative to this test, based on the colour change of the medium from blue to fluorescent yellow while no colour change indicated the absence of siderophore production.

**Cell wall degrading enzyme production**

**Cellulase activity**

A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye. The bacteria were grown on CMC (Carboxy Methyl Cellulose) agar containing (g L⁻¹) KH₂PO₄ 1.0, MgSO₄.7H₂O 0.5, NaCl 0.5, FeSO₄.7H₂O 0.01, MnSO₄.H₂O 0.01, NH₄NO₃0.3, CMC 10.0, Agar 12.0. The pH was adjusted to 7.0 with 1 M NaOH. The CMC agar plates were incubated at 37°C for 5 days to allow for the secretion of cellulase. At the end of the incubation, to visualize the hydrolysis zone, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1 M NaCl for 15 min. Clear zone around the colony was indicative of cellulase enzyme production.

**Chitinase activity**

A minimal salt medium containing colloidal chitin as sole carbon and energy source was used. The medium consisted of Na₂HPO₄, 6g; KH₂PO₄, 3.0g; NH₄Cl, 1g; NaCl, 0.5g; yeast extract, 0.05g, colloidal chitin 1.0% (w/v) agar, 15g and distilled water, 1000 ml and incubated at 30°C. Colloidal chitin was prepared by the method of Hsu and Lockwood from crab shell chitin (Sigma). Colonies showing zones of clearance against the creamy background were recorded as chitinase-producing PGPR.

**Protease activity**

Protease activity was determined by casein degradation in skimmed milk agar medium. An ability to clear the skimmed milk suspension in the agar plates was taken as evidence for the secretion of protease.

**In vitro Antagonistic activity**

The bacterial isolates were screened for the ability to inhibit *M. phaseolina* by employing dual culture method on PDA plates. Individually a loopful of 2 days old bacterial cultures grown in nutrient agar media was streaked on one side leaving 1 cm from the margin, and then 6mm disc of fungal pathogen culture was placed at the other side. Plates
Molecular characterization of isolates
The bacterial isolate which gave promising results in the preliminary test was grown in Luria broth for 24 hrs at 37°C for extraction of genomic DNA through enzymatic lysis\textsuperscript{15}. The integrity and concentration of purified DNA was determined by agarose gel electrophoresis. The total genomic DNA extracted was dissolved in water (protease, nuclease free) and stored at 4°C. PCR amplification was done by using 16S universal primer PA (5\textsuperscript{1} - AGAGTTTGATCCTGCTGCGAG\textsuperscript{3}) and PH (5\textsuperscript{1} AAGGAGGTGATCCAGGCGGA -3\textsuperscript{1}). The PCR (Peliter Thermal Cycler, BIO-RAD) reaction was performed in 100μl volume (10 μl of 10x PCR buffer, 2.5 μl of each primer, 1 μl of Taq DNA polymerase) with 3 μl of DNA Template. The amplification was performed with following program: 5 minutes initial denaturation at 95°C, followed by 30 cycles of 1 minute denaturation at 95°C, 1 minute annealing at 55°C, 1 minute extension at 72°C, and a final extension step of 5 minutes at 72°C. The amplified product was resolved in 1.2% agarose gel and visualized on gel documentation system (BIO- RAD, USA). The purified 16S rRNA gene was performed using as a template in cycle sequencing reaction with fluorescent dye- labeled terminators (Big dye, Applied Biosystems) of isolate each cluster with same primer and run in 3130 XL ABI prism automated DNA sequencer. The sequence was compared with 16S rRNA gene sequence available in the NCBI GeneBank database using BLASTn program.

RESULTS AND DISCUSSION

Isolation and Identification
An investigation was carried out to explore the multiple plant growth promoting activity and antagonistic activity of Rhizobacteria of sunflower crop. Totally ten bacterial species belonging to six genera were isolated and identified based on morphological and biochemical characteristics (Table-1). Similarly Raval and Desa\textsuperscript{16} isolated thirty bacterial species from rhizosphere soil of sunflower and also reported that these bacterial isolates increased the seed germination, root length and shoot length of Sunflower (Helianthus annuus L.) and other plants in pot culture experiments. The sequence of CCPS1 was compared with previously published sequence on the NCBI database and revealed high similarity to Bacillus amyloliquefaciens FZB42 (99% similarity), hence the isolate CCPS1 was identified as Bacillus amyloliquefaciens. This result supports the previous report of Mubarak et al.\textsuperscript{17} they have identified two potential chitinolytic strains namely Bacillus cereus and Bacillus sp using 16SrRNA sequence.

IAA production
Out of the ten isolates, 70% of isolates produced IAA in broth medium containing Tryptophan. Among the producers, it was noted that the Bacillus amyloliquefaciens was the strong producer of phytohormone IAA, while the remaining 60% of the test isolates were recorded as weak producer of IAA. The potential for auxin biosynthesis by rhizobacteria can be used as a tool for the screening of effective PGPR strains\textsuperscript{16}. Sajani Samuel and Muthukkaruppan\textsuperscript{19} studied the PGPR for their multiple growth promoting traits and reported that all the test isolates were able to produce IAA. Sasirekha Bakthavatchalu et al.\textsuperscript{,20} isolated 51 Pseudomonas spp from rhizosphere soils of different crops such as cabbage, wheat, potato, tomato, paddy and garlic. They reported that out of 51 isolates Pseudomonas aeruginosa exhibited promising results for IAA production, they also observed that the inoculation of cowpea seeds with this bacteria significantly
increased the seed germination, seedling vigor index, plant height, fresh and dry weight in comparison with the control.

**Siderophore production**

Siderophores are low molecular weight, iron chelating ligands synthesized by microbes. Siderophores help the microorganisms to compete against fungal pathogens for available iron and the role of siderophores in control of diseases has been well documented\(^2^1\). It has been suggested that siderophore producing bacteria could increase the germination power of agricultural plants.\(^2^2\) In this investigation 40% of isolates screened were positive for siderophore production. *Pseudomonas* sp2, *B. amyloliquefaciens*, *Pseudomonas* sp1, and *Micrococcus* sp1 produced clear yellow zone around the colony. Purified siderophore of *Pseudomonas aeruginosa* showed antifungal activity against *Aspergillus niger*, *A. flavus*, *A. oryzae*, *Fusarium oxysporum*, *Sclerotium rolfsii* \(^2^3\).

**Phosphates solubilization**

Phosphorus (P) is a major essential macronutrient for biological growth and development. Microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil and make it available to the plants. The ability of some microorganisms to convert insoluble phosphorus (P) to an accessible form, like orthophosphate, is an important trait in a PGPB for increasing plant yields\(^2^4\). High proportion of phosphate-solubilizing microbes is concentrated in the rhizosphere, and they are metabolically more active than other sources\(^2^5\). In our experiment all bacterial isolates were screened for phosphate solubilising capability. The results for phosphate solubilization showed that 40% of the bacterial strains were able to solubilize the tricalcium phosphate, as evidenced by hallow zone around the colony. Among the solubilizer *B. amyloliquefaciens* showed moderate solubilization, *Pseudomonas* and *Staphylococcus* showed weak solubilization. Efficient phosphate solubilizing bacterial species have been identified from the rhizosphere soil of onion, maize, jasmine, and tomato by Ranjan et al., \(^2^6\). Zarrin Fatima\(^2^7\) reported that the three strains of bacteria belonging to the genus *Azotobacter* isolated from Wheat rhizosphere possessed phosphorous solubilization and IAA producing characteristics. They concluded that these bacterial strains increased the seed germination, biomass and root, shoot length by inhibiting *Rhizoctonia solani* growth when tested in pot culture experiments. The phosphate solubilizing bacterial application was able to mobilize P efficiently in the sunflower and improved seed quality and oil yield. It also enhanced the head diameter, 1000 seed weight, kernel ratio and oil content and led to seed and oil yield increases of 15.0 and 24.7% over no application, respectively\(^2^8\). Manivannan et al.,\(^2^9\) isolated ten plant growth promoting bacteria from rhizosphere of rice and reported that among the ten isolates only one was found to be a phosphate solubilizer, also exhibited moderate (30%-40%) antifungal activity against *Fusarium oxysporum*, *Rhizoctonia solani* and failed to inhibit *Sclerotium rolfsii*.

**Ammonia production**

Production of volatile ammonia has been implicated as a possible mechanism to control soil borne pathogens\(^3^0\). In the present study 80% of the isolates were identified as ammonia producers, However *Pseudomonas* sp1 and *Bacillus amyloliquefaciens* were recorded as moderate producer. While the others, *Bacillus* sp1, *Bacillus* sp2, *Pseudomonas* sp2, *Neisseria*, *Micrococcus* sp1 *Micrococcus* sp2, were recorded as weak producers. The efficacy of ammonia producing PGPR has been well exploited by several previous studies\(^3^1\).

**HCN production**

HCN is produced by rhizobacteria and has been postulated to play a role in biological control of pathogens\(^3^2\). In the present study, we have observed that eight of our isolates produced HCN and among them two were strong producers (+++), one was moderate producer (+) and five were weak producers (+). This report supports the previous reports by Akhtar
and Siddiqui and Ruchi et al., The role of HCN in bicontrol activity of PGPR has been previously reported. Pseudomonas aeruginosa strain NJ-15 has been characterized as potential HCN producer which significantly inhibited the growth of phytopathogenic fungi in the order of Fusarium oxysporum > Trichoderma herizum > Alternaria alternata > Macrophomina Phaseolina.

Cell wall degrading enzyme production

In order to obtain carbon nutrition several biocontrol agents are able to hydrolyse chitin of fungal cell wall. Several studies have demonstrated the role of cell wall degrading enzyme on the control of pathogenic fungi. Kamil et al., have isolated effective chitinolytic bacteria belonging to the genus Bacillus namely Bacillus licheniformis and B.thuringiensis from the rhizosphere soil of maize, wheat and rice. Yildiz et al., screened and selected rhizobacteria against Fusarium oxysporum f.sp.melongenae from root zone of eggplant. Out of 261 isolates, eight of the isolates showed protease activity and no isolates showed chitinase and cellulose activities. Chaikharn et al., observed that 6% of rhizosphere isolates possessed cellulase and chitinase activity and 5% of rhizobacteria showed protease activity. Similarly, five of our isolates were screened for cellolytic activity, of which the strong producer was found to be Bacillus amyloliquefaciens. The same isolate showed moderate and strong activity of protease and chitinase respectively. 60% of rhizobacterial isolates exerted chitinolytic activity but only two species namely B. amyloliquefaciens, Bacillus sp1 showed very clear zone on colloidal chitin agar medium (Table - 2).

Antifungal activity against Macrophomina phaseolina

Neetu Singh et al., reported that Bacillus subtilis BN1 exhibited strong antagonistic activity against Macrophomina phaseolina, and other phytopathogens including Fusarium oxysporum and Rhizoctonia solani. The antagonist possessed the multiple plant growth promoting activity such as IAA production, phosphate solubilization, siderophore production. Similarly we have observed that the Bacillus amyloliquefaciens (35%) was the potent inhibitor of M. phaseolina, and also a good PGPR as it possessed the above stated characteristics. Pseudomonas sp1 (27%), Bacillus sp1 (30%) and Micrococcus sp1 (15%) also inhibited the mycelial growth but the rest of the isolates failed to inhibit the growth of pathogenic fungi. These results are in line with report of Usha and Padmavathi.
### Table - 1

**Morphological and Biochemical Characteristics of Rhizobacteria**

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Gram Reaction</th>
<th>Shape Isolates</th>
<th>Colony Colour</th>
<th>Motility</th>
<th>Indole</th>
<th>M.R.Test</th>
<th>V.P.Test</th>
<th>Citrate</th>
<th>Catalase</th>
<th>Bacterial Isolates</th>
</tr>
</thead>
<tbody>
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<td>CCPS1</td>
<td>+</td>
<td>Rod</td>
<td>Pale white</td>
<td>Motile</td>
<td>-</td>
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<td>-</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>Micrococcus sp2</td>
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+ Positive, - negative, MR-Methyl Red, VP, Voges Proskauer

### Table - 2

**Plant Growth Promoting and traits and antifungal activity**

<table>
<thead>
<tr>
<th>Bacterial Isolates</th>
<th>IAA production</th>
<th>NH₄⁺ production</th>
<th>HCN production</th>
<th>Siderophore production</th>
<th>Phosphate solubilization</th>
<th>Cellulase activity</th>
<th>Protease activity</th>
<th>Chitinase activity</th>
<th>Antifungal activity Zone of inhibition (%)</th>
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</tbody>
</table>

- Negative, + Weakly Produced, ++ Moderately produced, +++ Strongly Produced

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B - 183
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