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RESEARCH ARTICLE

ARBUSCULAR MYCORRHIZAL FUNGI AND OTHER BENEFICIAL MICROORGANISMS IN THE RHIZOSPHERE SOIL OF *GARCINIA LANCEIFOLIA* (G.DON) ROXB.

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ABSTRACT

A study was undergone to explore the soil beneficial microorganisms in rhizosphere soils of *Garcinia lanceifolia* (D.Don) Roxb. Rhizosphere soil samples were collected from ten different villages of Jorhat, Nagaon, Golaghat, Sivasagar and Dima Hasao districts, Assam. Beneficial microorganisms in rhizosphere soils of *Garcinia lanceifolia* were cultured, isolated and identified during 2016-17. The study exposed that *Garcinia lanceifolia* has Arbuscular mycorrhizal fungi (AMF) association in roots. Average root colonization of the plant species was recorded for 63.4 with a range of 50- 96 per cent depending on the place of collection. AMF spores were isolated from rhizosphere soils and identified tentatively up to the genus. AM fungi, nitrogen-fixing bacteria, phosphate solubilizing microorganism (PSM) and plant growth promoting rhizobacteria (PGPR) have been reported as useful for sustaining soil health and plant survival. Twenty-one (21) types of AMF spores were isolated from Garcinia lanceifolia rhizosphere soils belongs to eight (8) genus of 5 families of Glomeromycota i.e. Glomus, Acaulospora, Diversispora, Steptoglomus, Funneliformis, Rhizophagus, Entrophospora, and Gigaspora. Apart from arbuscular mycorrhizal fungi some other beneficial bacteria such as Fluorescent Pseudomonas and Bacillus spp were isolated from different culture media and identified. Some PSM, phytostimulator and decomposer microfungi such as Penicillium, Aspergillus, Trichoderma, Mucor etc were also isolated from rhizosphere soils of the plant species.

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INTRODUCTION

Garcinia lanceifolia (G.Don) Roxb. is a tropical evergreen plant grows as under shrub of family Clusiaceae. It has small thick epicarp fruit with a juicy, acidic, fragrant pulp. Leaves are lanceolate or elliptic, leathery. Flowers are cymes, creamy pinkish red, and blossoms from April to October. Fruit is berry, ovoid, orange-yellow in color, one-celled, and seeds up to five (enclosed in an edible aril-like pulp). The ripened fruits are eaten as chutney and mixed with curry. The popular traditional use of the fruit is in the preparation of sauce and cold drinks. Fruits are of high medicinal value and dried fruits are used for curing blood dysentery and indigestion and colic pain in the traditional system of medicine (Sarma *et al.*, 2010). The plant is used for treatment of headache (Paul *et al*., 2010); decoction of fruits is used in stomach ailments, leaves are used for treatment of stomachic, diuretic and the fruits are used for dysentery and diarrhea and flatulence (Buragohain, 2011). The fruit pulp is applied on wounds and decoction of fruit with salt is swallowed for fever. The crude fruit extracts were reported to be antibacterial leaves are used to treat stomachic diabetic, tender leaves are consumed as vegetable by some tribes (Gogoi *et al*., 2016). The methanolic extract of *G. lancifolia* flowers and fruits contains flavonoids, tannins, reducing sugar, steroid terpene and cardiac glycosides (Chowdhury and Handique, 2012). The plant species was recorded from evergreen forests of Lakhimpur, Sivasagar, Cachar, Nagaon, Khasi Hills, Garo Hills (Meghalaya) and Naga Hills (Nagaland) (Kanjilal *et al.*, 1991). Presently the plant species is reported as endangered and rare in wild condition and only occurring under cultivation in homesteads and generally grown under shade i.e. the crown cover of upper story trees (Rai *et al*., 2011). The rhizosphere is the soil surrounding to the root system of the plant. The rhizosphere is a good habitat of many microorganisms and they contribute in growth and survival of the plant species (Micallef *et al*., 2009; Chaparro *et al*., 2014; Sugiyama *et al*., 2014; Sun *et al*., 2014). Some rhizospheric microorganisms such as Arbuscular Mycorrhizal Fungi (AM fungi) are associated in the root of certain plant species and could contribute the plant by improving phosphorus nutrition (Smith and Gianinazzi-Person, 1988). Retaining in the root cortex, they behave like pseudo roots and involve in up taking of water, soluble minerals and micronutrient such as zinc, copper

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and sulphur by mycorrhizal root (Swaminathan and Verma, 1979; Li *et al*., 1991) and finally contribute in improvement of plant growth (Nouaim & Chaussod, 1994; Deelerck *et al*., 1995). Positive effect of inoculation of AM fungi along with other microorganism such as nitrogen fixing bacteria, phosphate solubilizing microorganism (PSM), plant growth promoting rhizobacteria (PGPR) has been reported as useful for sustaining soil health and plant survival in land use systems (Camprubi *et al*., 1995; Dutta, 2002; Hazarika *et al*., 2010a&b). Few bacterial species reported colonizing very efficiently the roots or the rhizosphere soil of the plant species (Kuzyakov & Domanski, 2000). These bacteria are referred to as plant growth promoting rhizobacteria (PGPR). In turn, the PGPRs perform some important activities for plant growth and health either by improving nutrient acquirement or by hormonal stimulation. Role of micro-organisms associated in rhizosphere are reported as imperative for plant growth, as they influencing nutrient cycling (Nayyar, 2009), favor the absorption of nutrients (Davison, 1988), produce hormones that promote growth (Denarie *et al*., 1992), fix nitrogen (Farnsworth, *et al*., 1977), suppress pathogens (Shippers *et al*., 1987), decomposition of organic matter in soils((Juma, 1998; Ruiter *et al*., 1994), dissolution of minerals (Nakas & Klein, 1980; Whalen and Hamel, 2004), suppress plant pathogens by production of antibiotics (Kent *et al*., 2002) and increase adaptive nature of plant species (Haney *et al*., 2015). PGPRs involve in enzymatic nutrient mobilization from organic matter and by the production of siderophores increase the availability of nutrients (Anderson *et al*., 1993; Whiting *et al*., 2001; Jing *et al*., 2007).

Some fungi such as Aspergillus, Penicillium, Trichoderma are reported to act either as Phyto-stimulator or inhibits the attack of rhizospheric pathogenic microorganisms and involve in decomposition of the organic matter, mineralization and release soluble form of nutrients to plant species (Tarkka *et al*., 2008, Brimecombe *et al*., 2007, Whipps, 2004, Vessey, 2003, Motsara *et al*., 1995). Plant-microbe interactions may thus be considered beneficial, neutral or harmful to the plant, depending on the specific microorganisms and plants involved and on the prevailing environmental conditions (Nihorimbere *et al*., 2011). Exploring these microorganisms by unraveling their possible relationships with plants has launched a new and fascinating area of investigations in the rhizosphere research. A considerable number of rhizosphere bacterial species are reported to exert the beneficial effect upon nutrient availability and soil aggregation (Johansen and Binnerup, 2002). Therefore, their use as biofertilizers or control agents for agriculture improvement has been a focus of numerous researchers for a number of years (Welbaum *et al*., 2004). *Garcinia lanceifolia* is an endangered edible fruit bearing species and the barks are reported to use to extract medicine (Bora *et al*., 2015). So far, there has not been any report for the study of rhizosphere soil beneficial microorganisms of *G. lanceifolia*. Therefore, the present study is undertaken to explore soil beneficial microorganisms of *G. lanceifolia* rhizosphere.

MATERIALS AND METHODS

Study site

The samples of plant species were collected from a total 10 villages of Jorhat, Sivasagar Nagaon, Dima Hasao and Golaghat districts of Assam viz Jalukoni, Titabor, SahpuriaGaon, Gohai Tekela Gaon, Selenghut of Jorhat district; Anaighoria Gaon, Morongial and Dholi Kumar Gaon of Golaghat district; Geleki of Sivasagar, Jokholabondha of Nagaon district and Jatinga of Dima Hasao during December, 2016 and January 2017. The study areas of these five districts are located in between $21^{\circ}30^{//}$ N to $26^{0}29^{//}$ N latitude and $92^{\circ}69^{//}$E to $95^{o}25^{//}$ E Longitude. Average annual temperature varies between 9°C to 39°C. The districts receive an average rainfall of 2244 mm per annum. The northwest monsoon is contributing a major share in governing the climate of the area.

Fig.1. *Garcinia lanceifolia* plant and flowering and fruiting and soil collection from the rhizosphere

Sample collection

Roots and soil samples were collected from near the base of the plant species up to 15 cms from the surface. There were three plants sampled for each plant species and the three samples were mixed to obtain one composite sample for analyses. Two such composite samples were taken for each plant species. This sampling procedure was repeated for each plant. Tendered roots of *Garcinia lanceifolia* were collected for investigation of AMF structures such as arbuscule, vesicle and mycelium along and to study root colonization by AMF of the individual plant species by tracing them horizontally from the base of the plant.

Clearing, staining and per cent AM colonization of root samples

The root samples collected were washed with tap water to remove the adhered materials, cleaned and kept in a hot air oven at 45° C for 72 h. Such dried roots of the samples were cut into 1 cm pieces, soaked in tap water, and kept in a 10 per cent KOH solution for 24 h. It was then washed in tap water for several times and bleached with 10% H_2O_2, slightly acidified with 0.01N HCl at room temperature and stained in 0.05 per cent Trypan blue in lectoglycerol (Phillips and Hayman, 1970; Kormanik and Mc Graw, 1982). After 24 h the roots were transferred to 50% glycerol and kept until per cent AM colonization determined. Stained root segments were examined under a stereo-compound microscope (Leitz-Laborlux 11 POL) at 100 x for AMF vesicles, arbuscules, hyphae etc. and hundred such root segments were examined for per cent AM

colonization. Per cent AM colonization was quantified by gridline intersect method (Giovannetti and Mosse, 1980). Root segments with AMF structure such as vesicles, arbuscules and hyphae with AMF spores on DPX mounted slides with clear structure were microphotographed.

Per cent root length colonization ten (10) numbers of root segments of 1 cm long were selected at random from a stained sample and mounted on microscopic slides. About 50 roots segments from each sample were used for slide method (Read *et al.,* 1976) and observed under microscope for presence and absence of AMF structure such as vesicles, arbuscules and hyphae with AMF spores.

Percent AMF colonization was calculated by using the formula

$$\% \text{ root colonization} = \frac{\text{No. of root bits having colonization}}{\text{Total number of root bits observed}} \times 100$$

Isolation, quantification and identification of AM fungal spore

Each triplicate soil samples were mixed thoroughly but separately and roots fragments separated. From each thoroughly mixed soil samples, two sub-samples of 100 g were weighed. One sub-sample was used for determination of spore number and other was oven dried at 60^{o} C for 72 h to calculate the number of spore in soil dry weight basis. AMF spores were isolated by wet sieving and decanting method (Gerdemann and Nicolson, 1963). 100 g soil sample was suspended in about 700 ml of water in a one-liter beaker and stirred for 3 to 4 minutes so that all soil particles were suspended. Soil and spore suspension were passed through a set of brass sieves of 460, 150, 120, and 45µm. These steps were repeated for three times and the spores along with debris retained on the sieves were collected in 100 ml beaker separately. The 500 m sieve found retained no spores or sporocarps except root fragments and other debris. The spore suspension collected in the beakers was transferred to watch glass with the help of pasture pipette and examined under a stereomicroscope and a stereo-zoom microscope. Apparently healthy spores with regular wall orientations were counted and similar looking spores separated. Isolated AMF spores will be examined thoroughly under the stereomicroscope.

Similar looking spores were transferred to separate vials, and detailed study for identification and classification was carried out. Spores selected for identification was mounted on polyvinyl lectoglycerol (PVLG) and identified up to genus and/or species level. Identification and documentation of an organism are done on the basis of its certain characteristic features include morphological, biological, ecological and even molecular traits. Primarily, AMF identification involves measurement and evaluation of spore characteristics. Morphological characters always play an important role in classification. Identification is also achieved by comparing individuals using preserved specimens, species descriptions, illustrations and keys. The morphological characters used for identification of AMF are organization of spores, sporocarp morphology, morphology of intact spores, spore wall characters, morphology of the subtending hyphae, occlusion of spores and morphology of soporiferous saccules (Morton, 1988). Gerdemann and Trappe (1974) first categorized AMF spores in loosely formed clusters, randomly dispersed in a loose or dense hyphal network or highly ordered with a hyphal plexus. The spores, after isolation can be separated under a stereoscopic microscope based on the characters such as color, size, shape, etc. Proper identification of each fungus requires careful examination of a number of spores under a light microscope, often at high magnification. Spores selected for identification were mounted on polyvinyl lectoglycerol (PVLG) and identified up to genus and / or species level with the taxonomic key in the manual for the identification of AMF by Schenck and Perez (1990) and INVAM (2015), (International collection of Vesicular-Arbuscular Mycorrhizal Fungi) Classification of West Virginia University, Florida.

Plant Growth- Promoting Rhizobacteria (PGPR)

Plant growth promoting rhizobacteria were isolated from rhizosphere soil of *Garcinia lanceifolia* by using serial dilution technique. 1 gm of soil sample was taken in the test tube containing 9mL of distilled water and shakes the test tube. Serial dilution was performed up to $10^{-1,}$, 10^{-2}, 10^{-3}, $10^{-4,}$ 10^{-5}, 10^{-6}, 10^{-7} dilution. Aliquots of 0.1ml suspension was transferred from 10^{-2}, 10^{-3}, $10^{-4,}$ 10^{-5}, 10^{-6}, 10^{-7} dilution on sterilized Petri plates. After that, 0.1 ml aliquot was transferred from each and every test tube by sterilized Petri plates to the agar plates and did spread plate in both King's B agar and Nutrient agar media. Inoculated Petri dishes were kept in the incubator at 27- 30ºC for 4-5 days. Glassware's used in the experiments were sterilized in hot air oven by exposure to steam at 121^{0}C and 15 for 15 minutes or longer, depending on the nature of the item. Almost all media were sterilized in the autoclave at 15lb pressure to attain the temperature of 121^{0}C.
The fluorescent *Pseudomonads* are a large group found in the rhizosphere of various plants. For culturing the Pseudomonas King's B medium was used the composition of the medium is given below. These rhizobacteria were purified by streaking on fresh plates (Streak plate method) containing King's B medium and preserved at – 30^{o}C for subsequent inoculation. Streaks were made across the surface of the agar with a loop full (inoculating loop) of mixed culture. After inoculation, the Petri plates were incubated at 28 to 30^{o}C for 2 to 3 days and when the bacterial colonies formed, the Petriplates were observed under florescent/ ultraviolet light. Yellow-green or blue-white fluorescence in and around the colonies were seen for fluorescent *Pseudomonas*. The bacterial mass from the single/ pure colony was taken with a needle from its periphery and put on the glass slide and stained with Gram stain and mounted by a cover slip. The size and shape of the bacterial cells were observed under the stereo-compound microscope at 40X and followed by100X using Emerson oil and distinct morphology of the bacterium was microphotographed.

Culture of *Bacillus* spp

Bacillus spp were isolated from the rhizosphere soil of *Garcinia lanceifolia* with the help of serial dilution technique (following the same procedure described for *Pseudomonas florescent* mentioned above). Spread plate method was followed for the culture of *Bacillus* species in Nutrient Agar medium by transferring 0.1 aliquots of each dilution. The bacterial mass from the single pure colony was taken with a needle from its periphery and put on the glass slide and stained with Gram stain and mounted by a cover slip. Morphological characterization was done as per procedure describes for

Florescent Pseudomonas observed under the compound microscope.

Culture of rhizospheric fungi

1 gm soil collected from the rhizosphere of *G. lanceifolia* was taken and undergone the serial dilution techniques and different dilutions were taken for culturing the microfungi in Potato Dextrose Agar (PDA) medium. Sterilization of culture media, glassware, and aseptic conditions were maintained. Autoclave, hot air oven, laminar air and antibacterial agent were used for maintenance to free other contaminations. Mycelium and spore-bearing hyphe from axenic culture were observed under the microscope for their characterization and micro photography.

Statistical analysis

No other statistical analysis are performed except the calculation of the average of the AMF spore number 100 g-1 soil and per cent root colonization of 10 different sites of the soil sample collected.

RESULTS

Arbuscular mycorrhizal colonization in roots and AMF spores extracted from the rhizosphere soils

Arbuscular Mycorrhizal fungi colonization in roots

The microscopic observations under the stereo-compound microscope revealed the presence of vesicles, arbuscules and extramatrical mycelium in cortical zones of the plant roots are presented in the Figure-2 and Table-1. The average per cent root colonization of the plant species was recorded for 63.4. It was also observed that the per cent root colonization could vary with the place to place. It ranged with a minimum of 50 to a maximum of 96 per cent.

Table 1. Percent root colonization of arbuscular mycorrhizal fungi in root of *Garcinia lanceifolia*

Place of collection	Total root bits	Colonized root bits	% root colonization (colonized bits/total root bits observed ×100)
Dholi Kumar Gaon, Jorhat	50	25	50
Jatinga, Dima Hasao	50	26	52
Anaighoria Gaon, Golaghat	50	30	60
Morongial,Golaghat	50	32	64
Jalukoni, Titabor, Jorhat	50	27	54
Sahpuria Gaon, Jorhat	50	30	60
Selenghut, Teok, Jorhat	50	40	80
Jokholabondha, Nagaon	50	27	54
Gohai Tekela Gaon, Jorhat	50	32	64
Geleki, Sivasagar	50	48	96

AMF spores in rhizosphere soils

Garcinia lanceifolia rhizosphere soils contained about 21 types of AM fungi spores of eight (8) genus (Fig-3).Of which, serial No. A-F represented *Glomus* spp., G-K: *Acaulospora* spp., L&M: *Diversispora* spp., N: *Steptoglomus* sp., O: *Funneliformis* sp., P& Q: *Rhizophagus* spp ., R: *Entrophospora* sp. and S-U : *Gigaspora* spp. It was also recorded that all soils samples did not represent the same number of AMF spores. The minimum number of AMF spores were detected from Dholi Kumar Gaon with 104 spores $100g^{-1}$ soil. The maximum numbers of AMF spores were isolated from Anaighoria Gaon, Golaghat with 610 spores $100g^{-1}$ soil. However, arbuscular mycorrhizal spores types were almost similar to all soil samples studied invariably to the site of collection (Table-2).

Table 2. AMF spores $100g^{-1}$ isolated from rhizosphere soils of *Garcinia lanceifolia*

Place of Collection	No. of AMF spores $100g^{-1}$ soil	Average number of AMF Spores
Dholi Kumar Gaon	104	
Jatinga, Dima Hasao	299	
Anaighoria Gaon, Golaghat	610	
Morongial,Golaghat	330	
Jalukoni, Titabor, Jorhat	310	
Sahpuria Gaon, Jorhat	450	358
Selenghut, Teok, Jorhat	397	
Jokholabondha, Nagaon	205	
Gohai Tekela Gaon, Jorhat	495	
Geleki, Sivasagar	375	

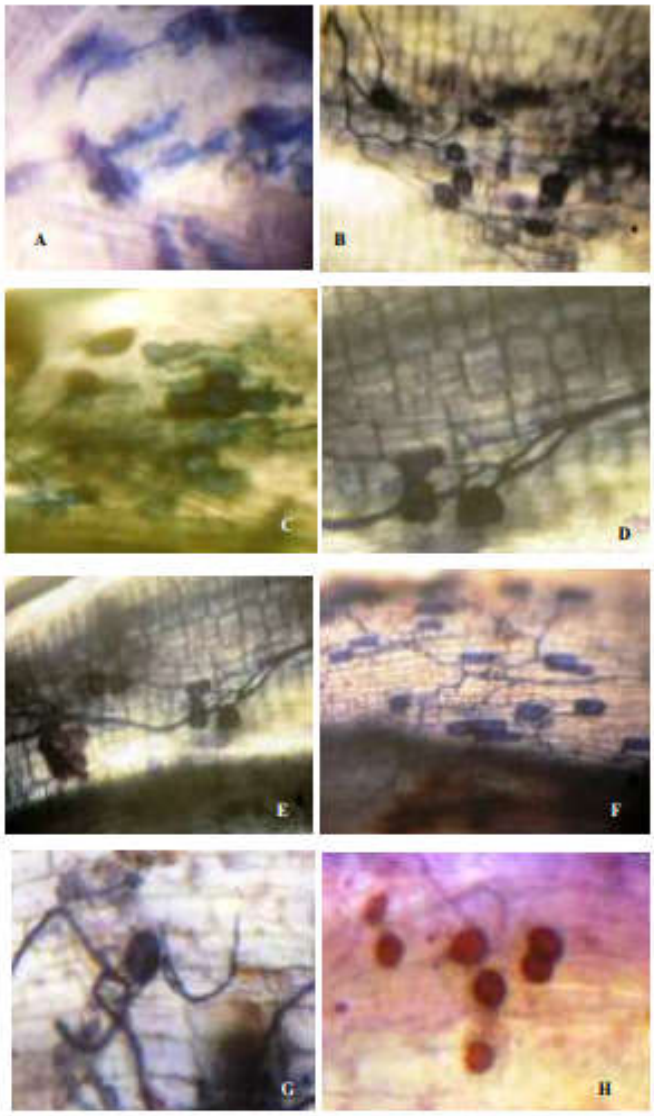

Fig.2. Microphotographs of arbuscular mycorrhizal fungi structures in root cortex of *Garcinia lanceifolia*. [A]- arbuscules, [B-F] –different types of vesicles, [G]- mycelium with spore and [H]- AMF spores inside the root cortex

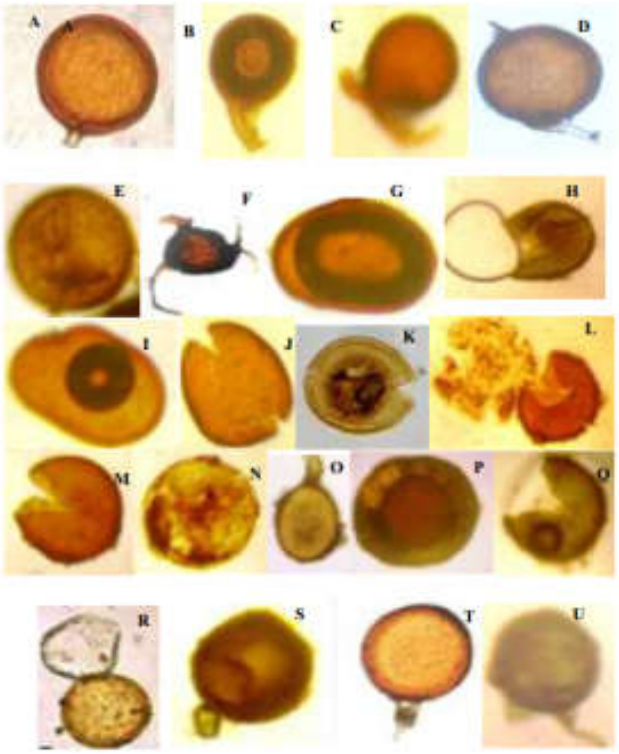

Fig.3. Photomicrographs of Arbuscular Mycorrhizal Fungi spores isolated from rhizosphere soils of *Garcinia lanceifolia* A-F: *Glomus* sp., G-K: *Acaulospora* sp., L&M: *Diversispora* sp., N: *Steptoglomus* sp., O: *Funneliformis* sp., P& Q: *Rhizophagus* sp ., R: *Entrophospora* sp. and S-U: *Gigaspora* sp.

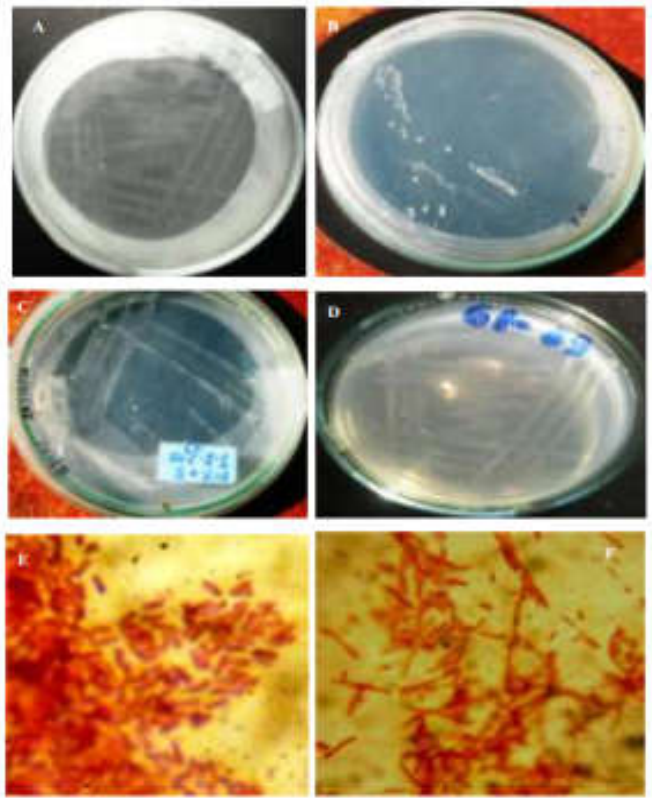

Fig.4. Photographs of Bacterial growth n culture media. [A-D] Fluorescent Pseudomonas in King's B medium. [E]- Microphotograph of Fluorescent Pseudomonas sp., [F]- Bacillus sp., Isolated from rhizosphere soils of *Garcinia lanceifolia*

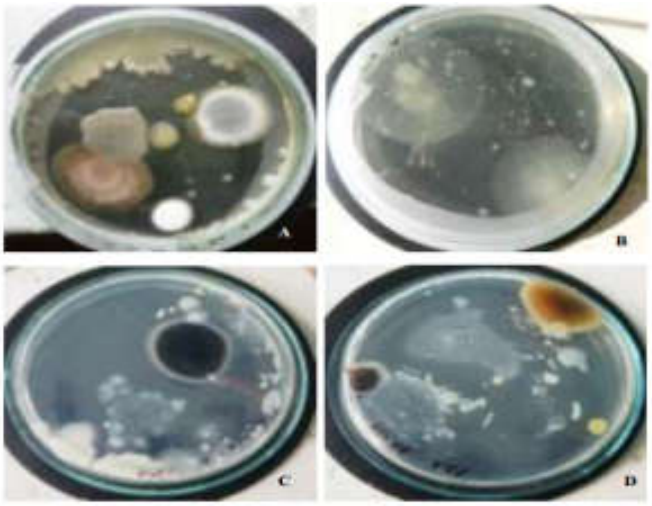

Fig.5. Photographs of fungal colony formation in PDA media. [A-D] cultured from rhizosphere soils of *Garcinia lanceifolia*

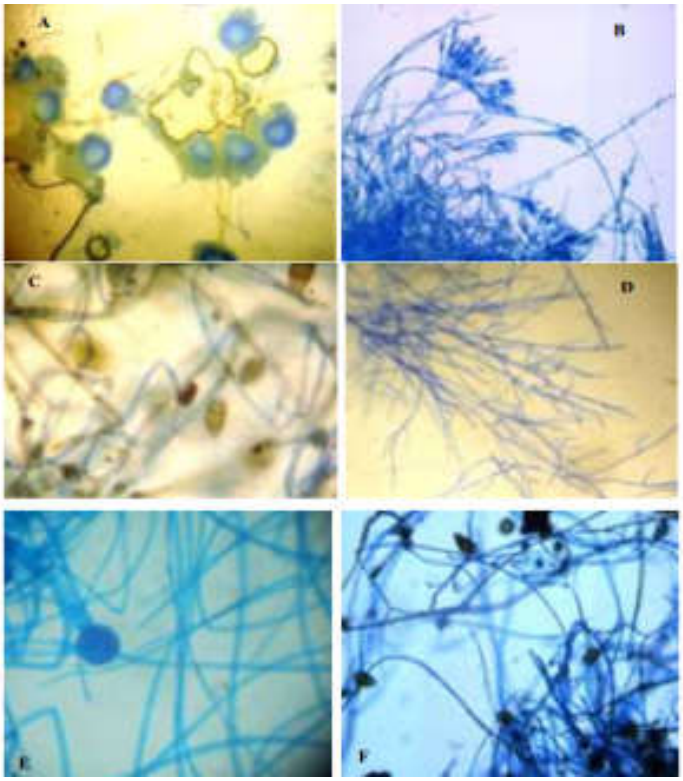

Fig.6. Photomicrographs of microfungi isolated from rhizosphere soils of *Garcinia lanceifolia* in PDA culture [A] *Aspergillus* sp., [B] *Penicillium* sp., [C] *Bipolaris* sp., [D] *Trichoderma* sp., [E] *Mucor* sp., [F] *Curvularia* sp.

Other beneficial microorganisms in rhizosphere soils of *Garcinia lanceifolia*

The culture of rhizosphere soils of the plant species observed the growth of bacterial colonies in Petri plates in dilutions of 10^{-2} to 10^{-7} in inoculated aliquots in King's B and Nutrient Agar media. Numbers of bacterial colonies counted in different dilutions i.e. 10^{-1} was 139, 10^{-3} had 132 10^{-4} had 78, 10^{-5} 40, 10^{-6} 30 and 10^{-7} contained only 28 bacterial colonies. The bacterial structures that observed under the stereo-compound microscope and the microphotographs were shown in fig. 4. Some micro fungi that were observed to grow in potato dextrose agar medium and photographs of them were presented in fig 5. Growth characteristic of the fungal colonies were

observed as light blue for *Penicillium* spp, Darty/ black colonies for *Aspergillus* spp and Cottony to brown for *Mucor*, *Biplories*, *Curvularia* spp and white to brown for *Trichoderma* spp. Microphotographs of microfungi isolated from rhizosphere soils of *G. lanceifolia* in PDA culture were presented in figure-6. Six microfungal genera such as *Aspergillus*, *Penicillium*, *Bipolaris, Curvularia*, *Trichoderma* and *Mucor* were isolated and identified.

DISCUSSION

Microorganisms are indispensable constituents of soil and they have direct or indirect influence in maintaining the soil's health throughout their beneficial or detrimental activities. Rhizosphere inhabiting microorganisms arbitrate soil processes such as decomposition, nutrient mobilization and mineralization, storage release of nutrients and water, nitrogen fixation and denitrification. Furthermore, the organisms possessing a phosphate-solubilizing ability can also convert the insoluble forms phosphate compounds into soluble forms in soil and make them available to the plant species. The study revealed that plant species could prefer good numbers of beneficial microorganisms. The plant and microbe interaction influences the plant growth and survival. Several factors may influence the survival of a plant species including anthropogenic pressure, habitat loss, and degradation of micro and macro environments including soil beneficial microorganisms. The rhizobacteria specifically fluorescent *Pseudomonas* and *Bacillus* spp and arbuscular mycorrhizal species associated with the rhizosphere of *G. lanceifolia* may also receive special attention to the researchers to find out the any relation with the cause of declination of plant species from the natural habitat. The study for soil microorganisms in rice rhizosphere from different locations in South Karnataka has reported a total of 57 Gram positive Bacilli, 6 Gram negative Bacilli, 47 Gram positive Cocci and six Actinomycetes (Ritesh *et al*., 2014). In comparison to rice rhizosphere least number of beneficial microorganisms was detected from *Garcinia lanceifolia* rhizosphere. In another study reported by Gnanasekaran *et al*. (2015) from the banana field of Manachanallur, Tiruchirappalli was found dominated by genera of fungi such as *Aspergillus*, *Penicillium* and *Trichoderma* species. The present study also revealed the similar results. This may also happen that the populations and diversity of soil beneficial microorganisms required sustaining the growth and survival of the *G.lanceifolia* not at par to give proper support to the plant species. Few works reported that processes shape and drive the composition and dynamics of the rhizosphere microbes not only to safeguard plant productivity but also to safeguard human health as the proliferation of human pathogenic bacteria in and on plant tissues (van der Heijden *et al*., 2007; Tyler & Triplett, 2008; Teplitski *et al*., 2011; Kaestli *et al*., 2012). The work done to recommend the proper solution for sustain this endangered plant species is not enough and can be considered as peeping through a window to see the room. Therefore, a detail research work is required to explore the actual cause (s) of loss of natural populations and to recommend the solution for the conservation of the plant species in the natural habitat.

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