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Diversity of Actinomycetes in Nitrogen Fixing Root Nodules of *Casuarinaequisetifolia* and its Impact on Plant Growth

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ABSTRACT

Casuarinaequisetifolia (Kasa) is an actinorhizal plant which is used for rehabilitation of poor and disturbed soil throughout the world. Actinorhizal plants which were colonized by Frankia sp. enhance the soil fertility due to frankial colonization in root nodules. However, few studies were reported on non-frankial colonization and its impact on plant growth and soil fertility. Thus this study was carried out to investigate the unrevealed information on actinomycete consortia residing the nitrogen fixing root nodules of C. equisetifolia. The actinomycete were successfully isolated from surface sterilized root nodules by using double layered agar plate technique and also isolates were tested on reinfectivity on Casuarina seedlings. Three different actinomycetes were able to identify as Frankia sp., Micromonospora sp., and novel symbiont as Streptomyces sp. from nitrogen fixing root nodules of Casuarina plants. Further, co-existence of all three isolates were observed in nitrogen free and nitrogen enriched Yeast Mannitol Agar medium (YMA). Frankia sp. promoted the shoot and root growth by 87% and 55% respectively with nodulation of C. equisetifolia and whereas Micromonospora sp. promoted the root and shoot growth by 28% without nodulation. In contrast Streptomyces sp. was able to trigger the lateral root formation of C. equisetifolia which indicate the ability of the microorganism to alter host development system. This study provides novel data on root inhabiting Streptomyces sp. which could play a vital role in enhancing plant growth, exchanging complex signals between plant and microorganisms. These results suggest that the root inhabiting microbial consortium of C. equsetifolia would significantly contribute to the development of plant growth.

KEYWORDS:*C. equisetifolia, Streptomyces, Frankia, Micromonospora, soil fertility*

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1. INTRODUCTION

C. equisetifolia is one of the most important actinorhizal plants which are used for the rehabilitation of infertile and disturbed soils to improve their stability and fertility. In addition, these plants have been frequently used in agroforestry, coastal protection and natural ecosystems.

Previous studies have reported that these actinorhizal plants mainly have a symbiotic association with Frankia sp.. Further, it has also been reported that Micromonospora sp. colonized in root nodules of C. equisetifolia (Valdes et al., 2005).

Several different non-frankiaactinomycetes were isolated from actinorhizal plants including Coriaria (Trujillo et al., 2005), Discaria (Solans et al., 2011), Alnuselaeagnus (Gtari et al., 2004). These associations have been assumed to play a vital role in both plant growth and soil quality (Echbab et al., 2004).

Solans et al., (2011) showed that Micromonospora sp., Streptomyces sp., Actinoplanes sp., enhanced the plant growth and nodulation frequency of Discariatrinervis.

Further, these actinomycetes produced three different phytohormones: indole-3-acetic acid (IAA), gibberellic acid (GA 3), and zeatin at higher levels than those produced by the symbiotic Frankia strain BCU110501 (Ghodhbane-Gtari and Tisa, 2014).

Little is known about non-frankiaactinomycetes and their associations with C. equisetifolia plants. Some of predicted roles of non-frankia associations are nitrogen fixation, helper bacteria hypothesis and plant hormone production, antagonism model.

Thus, it is essential to study about yet unrevealed information about microbial diversity and their beneficial roles on Casuarina plants.

The present study therefore was undertaken to investigate the microbial diversity in nitrogen fixing root nodules of *Casuarina* plants. Isolation and identification were based on morphological characteristics and microbial growth on synthetic media. Plant assays were also conducted to investigate the ability of isolates to form nitrogen fixing nodules and their effect on plant growth.

2. MATERIALS AND METHODS

2.1. Collection and maintenance of plant material

Casuarinaequisetifolia plants with nitrogen fixing root nodule were collected from plantation of National Forest Department, Rediyagama, Hambanthota.

2.2. Isolation and identification of nodule inhabiting actinomycetes

i. Surface sterilization of root nodules

Approximately 10 – 15 active root nodules were collected and carefully washed them with running water. The washed nodules were surface sterilized by following a surface sterilization protocol described elsewhere with some modifications (Gomma*et al*., 2008). In brief, a mixture of 30% H_2O_2 + 70% ethanol (1: 1 ratio) was used for surface sterilization.

Each nodule was fragmented into individual lobes and placed them in the mixture of 30% H_2O_2 + 70% ethanol (1: 1 ratio) for 20 minutes. Then lobes were transferred in to 1% sodium hypochlorite and kept them for 5 min. They were washed several times with sterile distilled water.

ii. Isolation of actinomycetes

The BAP medium and Yeast Mannitol Agar (YMA) were used to isolate actinomycetes (Vincent (1970). Double layered agar plate

technique was used for the isolation of actinomycetes from surfaced-sterilized root nodules (Diem *et al*., 1982).

Some surface sterilized lobes were directly placed on the bottom layer of the plates and then autoclaved 1.5% agar at around 45 ^{0}C was poured on it.

In addition, macerated nodule suspension was also streaked on the surface of the bottom layer of the plates and covered it with 1.5% agar as described above. All the inoculated plates were incubated in a candle jar 28 ±1^{0}C until microbial growth.

iii. Identification of isolated actinomycetes

Colonies were observed under light microscope (10x4) (Optika B-350, Japan). Filamentous cells of isolates and chains of spores of one isolate were observed under light microscope after staining them with 1% (w/v) lacto phenol cotton blue (10x40, 10x100).

iv. Determination of inhibitory growth of the isolates in a co-culture

The YMA plates were divided in to three equal slots and isolated microorganisms were inoculated on divided slots separately. The plates were incubated at 28±1 °C. Growth of all isolates together was observed.All the isolated cultures were preserved in 70% glycerol stocks at -80 °C until use.

2.3. Plant assay

Collected *Casuarina* sp.seeds from Hambanthota plantation were surface sterilized with 50 mL of 5% sodium hypochlorite (Chlorox) with 2 drops of Tween 80 for 5 min. Surface sterilized seeds were aseptically transferred on to sterile moist cotton wool and kept at 25 °C under dark condition until germination.

Four third volumes (¾) of fifty milliliter culture tubes filled with quarter strength Hoagland solution were used for the plant assay.

Germinated seeds were first grown in quarter strength N-enriched Hoagland solution for two weeks and then two weeks old seedling were transferred to culture tubes filled with quarter strength N-free- Hoagland solution. Seedlings were inoculated (5%) separately with exponentially grown cultures of each isolate.

Three replicates were maintained in each treatment and all the treatments were arranged in a completely randomly design (CRD) in a green house. Plants were observed for nodule formation, root growth and lateral root formation. Root and shoot length of 2 ½ month old plants were recorded.

2.4. Statistical analysis

Statistical analysis was carried out by using Mini Tab 16 software (version 16.2.4.0, minitab Ltd (UK), 2014).

3. RESULTS

Isolation and identification of root inhabiting actinomycetes from nitrogen fixing root nodules.

A good growth of three different actinomycetes was observed in double layered agar plates, which were incubated at 28 °C for 10-15 days. All isolates were found to be Gram positive and showed filamentous growth. One isolate showed or exhibited starfish shape white colonies on BAP medium (Figure 01: A).

Few isolates were found to develop orange-pigmented colonies on YMA plates incubated at 28 °C after 1 week. Another type of actinomycete was observed in white with aerial mycelia and black pigmentation on YMA plates incubated at 28 °C after 2-5 days under microaerophilic conditions.

Further showed a similar characteristic such as heaped, velvety looking colony characteristics to that of genus Streptomyces (Figure 2: A and B). Light microscopy was conducted to identify distinctive morphological characteristics of cells of three isolates when grown in synthetic media. *Frankia* sp. showed branched and septate hyphae with diazovesicles (Figure 01: B and C).

These diazovesicles were observed in *Frankia* sp. when it was grown in both solid and liquid medium of BAP, after incubation at 28 °C for 2 weeks. However, the density of diazovesicles was found to be high when it was grown inN-free liquid medium.

The observed orange color colonies showed branched, septate hyphae without forming any diazovesicles which is similar to that of genus *Micromonospora* (Figure 01: D).

Mycelia of *Streptomyces* sp. were found to be more abundant on surface of the YMA plates. Sporulation found to occur on aerial mycelia and chains of spores were produced after incubation under microaerophilic conditions at 28 ^{0}C for 5 days in YMA.

Growth rate of *Streptomyces* sp. were found to be high when it compared to those of, *Frankia sp.* and *Micromonospora* sp. which were grown in liquid medium of YMA incubated at 28 °C for 5 days.

Dry cell weight of *Frankia* sp. was found to be low out of three isolates in N-enriched the medium. *Frankia* sp. showed the lowest growth rate while *Streptomyces* sp. showed the highest growth rate in Yeast Mannitol broth (Table 01).

Similar growth patterns of isolates were observed in N-free liquid yeast mannitol broth. The ability of the isolate to grow in the presence of different cadmium concentrations (within the range of 0-10 mg/L) incorporate yeast mannitol broth was used to differentiate different isolates in terms of their tolerance limits to cadmium.

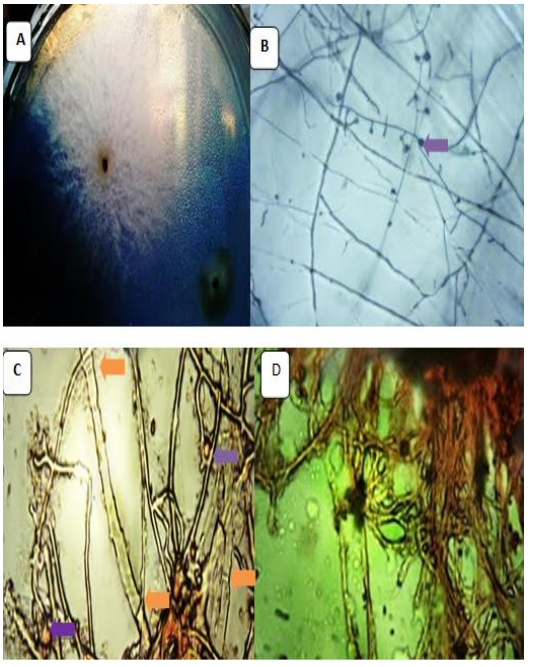

Figure 1. (A-C): Microscopic features of *Frankia* sp. and *Micromonospora* sp. isolated from surface sterilized root nodules of *C. equisetifolia.* A) *Frankia* sp. colony on BAP solid medium. B and C) *Frankia* diazovesicles on septate mycelia (marked with arrows in orange) and D) diazovesicles of (marked with purple arrow)*Micromonospora* sp.

Ability of formation of nodules by three isolates was studied in a plant assay. Nodulation was observed only in*Frankia* sp. treated seedlings. *Streptomyces* sp. treated seedlings showed increased level of lateral root formation when compared to that of other untreated seedlings.

Plant growth promoting activities of each isolates were determined by measuring the shoot/root length and height of seedlings. The data was shown in the Table 01.In current study, the seedlings which were treated with *Frankia*sp. showed the highest root (12.4 ± 0.80 cm) and shoot length (19.3 ± 1.06 cm) when compared to those of other treated and control seedlings. Data showed that along with different treatments, the plant shoot and root growth increased significantly ($P<0.005$).

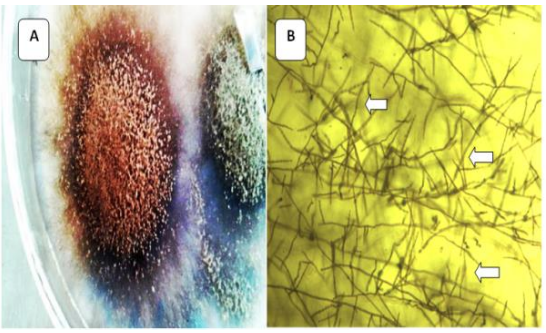

Figure 2.(A-B): Light microscopic view of morphological characteristics of *Streptomyces* sp. isolated from surface sterilized root nodules of *C. equisetifolia.*A) Growth of *Streptomyces* sp. on YMA plate. B) Spore chains of *Streptomyces* sp. on YMA plate (White arrows-spore chains)

Table 1. Effect of isolated actinomycetes on growth of shoot and roots of *Casuarinaequisetifolia*seedlingsgrown in Hoagland's solution

Treatment	Shoot length (cm) Mean ± SE	Root length (cm) Mean ± SE
Control	10.3±1.02	8.0± 0.96
Frankia	19.3 ± 1.06	12.4 ± 0.80
Micromonospora	13.1 ± 0.99	10.1 ± 0.94
Streptomyces	10.3 ± 1.67	9.5 ± 1.50

SE- Standard Error

Seedlings which were treated with *Frankia* sp. showed highest root and shoot elongation and *Streptomyces* sp. inoculated seedling showed the triggering effect of lateral root formation (Figure 3:C).

Co-existence activity was observed in triple and dual co-cultures of *Frankia* sp. with *Streptomyces* sp., *Micromonospora*sp. with *Streptomyces* sp. and *Frankia* sp., *Micromonospora* sp. and *Streptomyces* sp. respectively in YMA media. Inhibition zones were not observed in any of the plates.

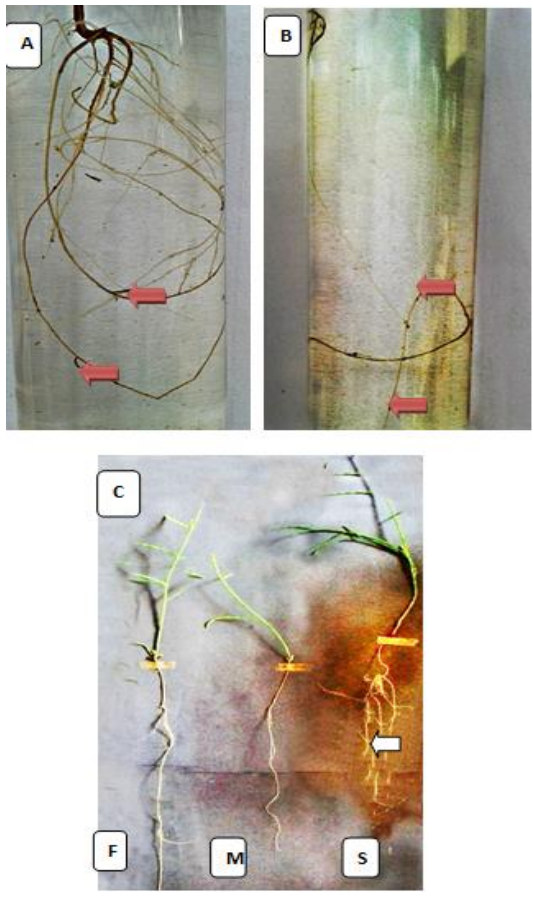

Figure 3. A and B) Nodule primodia of seedling treated with *Frankia* sp.arrows in red) C) Triggering effect of *Streptomyces* sp. on *Casuarina* seedlings (F-*Frankia* sp., M-*Micromonospora* sp., S- *Streptomyces* sp.

4. DISCUSSION

All three of isolates were Gram positive and showed filamentous growth which is one of major characteristics of Actinomycetales. *Frankia* sp. showed white star shape colony with septate, branched mycelia with diazovesicles on BAP solid medium plates. The vesicles are the most definitive structure of characterizing the Frankia genus (Benson and Silvester, 1993). Vesicles are normally initiated only in the presence of a nitrogen free medium or during growth on certain nitrogen sources, such as some amino acids, that cannot be degraded to simpler form, ammonia (Zhogz*et*

al., 1986). The vesicles initially develop as terminal swellings on hyphae or on short side branches. In this study considerable amount of vesicles which were formed in short side branches were observed in growth in BAP solid and liquid media.

As reported by Carro*et al*, (2013) some isolates developed orange colonies which are resembled to the typical characteristic of *Micromonosporaceae.* Similar results were observed in this study with raised orange colonies on YMA solid and Yeast Mannitol broth. *Micromonospora* sp. showed branched, septate, hyphae without diazovesicles. Colonies of *Streptomyces* sp. showed chalky, heaped folded with aerial and substrate mycelia on YMA medium.

The reasonable explanation for the slow growth of *Frankia* sp. is their generation time has reported as 15 h or more according to the study Benson and Silvester, (1993). Kawamoto *et al.*, (2014) have shown that *Micromonospora* sp. has doubling time as 7-8 h and it was as 4-6 h for *Streptomyces* sp. In present study high number of vesicles was observed in nitrogen free media as compared to that of in nitrogen enriched medium. The explanation for this result can be supported with facts of the study carried by Zhogze*et al.*, (1986), removal of NH_4^+ from the culture medium resulted in vesicle differentiation. The growth rate under atmospheric N_2 and medium supplied carbon source going to lengthen it's doubling time for 24 h. It has been reported by Murry*et al.*, (1984) that when nitrogen was withdrawn from medium growth of the mycelium arrested and initiated vesicle differentiation.

Seedlings which were treated with *Frankia* sp. showed the nodule formation ability out of three isolates. Further, the plants which were treated with *Frankia* sp. showed the highest shoot and root development. In contrast seedlings, which were treated with *Streptomyces* sp. showed the increased lateral root formation than that of other plants. This study was first reported on *Streptomyces* sp. colonization on nitrogen fixing root nodules of *Casuarinaequisetifolia*where *Frankia*sp. and *Micromonospora*sp. are alreadyknown as inhabiting actinomycetes in nitrogen fixing root nodules.

This tripartite symbiosis could be revealing a new path on *Casuarina* plant growth. Echbab*et al*., 2004 shown that the *C. cunninghamiana* plant growth promotion according to three hypotheses as an increase in phyto-hormones responsible for root length enhancement, Development of mycorrhizea, synergistic effect on *Frankia* growth. Diagne*et al*., 2013 shown that *Casuarina* plant roots colonized with endo /arbuscular (AMF) and ectomycorrhizzea (ECM) which increase the nutrient absorption capacity via their extensively distributed network of hyphae. Further they have shown that both AMF and ECM increase the plant resistance to diseases by forming external barrier and restrict the entering of the pathogen on to the plant, and alsoconfer resistance to drought condition. Previous studies have reported that inoculation with *Frankia* increase N_2 fixation by 15-80%, ectomycorrhizal colonization by 10-70% and endomycorrhizal colonization by 10-50% with EMF. *Micromonospora*sp. able to synthesize secondary metabolites that able to inhibit the growth of pathogens like *Pythium* and *Phytophthora*(Diagne*et al*., 2013). This emphasize that the importance of these root colonized actinomycetes on the growth and promotion.

The synergistic effect was shown by all two and triple cultures *in vivo*. Inhibition zones were not observed in co-cultures, even with well-known antibiotic producing bacteria *Streptomyces* sp.. This result emphasized that there is a synergistic growth or no inhibition within isolated actinomycetes consortia. Similar results have been reported by Solans*et al.*, (2011) that *Ochetophilatrinervi* plants, an actinorhizal plant inoculated with *Frankia* sp. showed high growth rate and length when plants were treated with *Streptomyces* sp. than control.

It can be concluded from this study that actinomycetes consortia residing the nitrogen fixing root nodules of *C. equsetifolia* able to play a vital role in enhance the plant growth and soil fertility. Further study on effect of different pH values, water holding capacity, acidity, and salinity on growth of isolated actinomycetes will improve the usage of *Casuarina* plant in agroforestry.

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