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Nodular Micro-Endosymbiontic and Endomycorrhizal Colonization in *Elaeagnus latifolia* L.: A noble host of Symbiotic Actinorhizal and Endomycorrhizal Association

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Abstract: ***Elaeagnus latifolia L. belongs to Elaeagnaceae family is uncommon in the North- Eastern states of India. In this study, the target plant species was screened for rhizospheric microbial association for the first time and it is found that it is in a symbiotic relationship with the nitrogen fixing Actinomycetous bacteria, Frankia sp. in root nodules. Moreover, the rhizospheric root samples were also having all the three types of root infection/colonization namely hyphal infection (90%), arbuscular infection (70%) and vesicular infection (25%). The qualitative analysis revealed that the predominant endomycorrhizae in the rhizosphere belongs to three genera viz. Glomus, Acaulospora and Gigaspora. Altogether, 13 species of AM fungi were observed. The diversity of Arbuscular mycorrhizae was retained by Acaulospora spp. (46.6%), followed by Glomus spp. (40%) whereas the occurrence frequency of Gigaspora spp. (13.3 %) was found to be the least among all the three genera.***

Keywords: Endomycorrhiza, Actinorhiza, *Frankia* species, Nodular Micro-endosymbiont, Rhizosphere

1. Introduction

Symbiosis is an ecological interaction in which two or more species live in close association with each other. Endosymbiosis is when an organism actually lives within another organism. This is also mutualistic symbiosis where the two partners living in close association with each other derive benefits from each other in terms of their ability of the partner to survive and reproduce [1]. Thus, symbioses between higher plants and bacteria or fungi are important and essential for better plant growth and development. A large number of microsymbionts have been identified such as legumes-Rhizobium, VAM- roots of higher plants; Frankia-Alnus, Allo-casuarina, Elaeagnus, Hippophae, Purshia and Shepherdia [2]. Among all these interactions, Frankia-Actinorhizal is inimitable and quite intriguing due to the similarities and differences these symbiotic interaction bear with that of the Rhizobium-legume system [1].

Elaeagnus latifolia L., a member of the family Elaeagnaceae, order Rhamnales also known as bastard Oleaster or *Myrica tenga* (Assamese) is uncommon in the north-eastern states of India. This plant species is economically valuable due to the wide usage of the fruit pulp of this small tree for making jam, jelly and refreshing drink. However, recent studies have shown that the density of these plant species have been lowered in the natural forest stands and the fruit collection procedure was highly erratic, threatening its survival in near future [3]. Thus, such fruit trees are in great need of conservation in their natural forest stands. Keeping this aspect in consideration, the target species was screened for rhizospheric microbial association as it has been established that microbial interactions can drive ecosystems functions such as plant diversity, productivity and variability.

2. Materials and Methods

2.1 Collection of plant, soil and root samples

The plant species was collected from four sites *viz.* Chekanidhara gaon, Balisapori gaon, Sotai and campus of Rain Forest Research Institute of Jorhat district (Fig.1) **(26.75°N – 94.22°E).** Rhizospheric soil samples were collected by digging out a small amount of soil (500gm) close to plant roots up to the depth of 15-30 cm and further processed in the laboratory for analyses of endomycorrhizal and actinorhizal microsymbiotic associations.

Figure 1: Map showing the study site in Assam, India

2.2 Isolation of Bacterial microendosymbiont

For isolation of the bacterial microendosymbiont, the method employed by Gomaa et al. [4] was used. A number of active nodules of the target plant species were selected and then washed several times in tap water and then with distilled water to remove the soil particles from the nodules. The nodules were segmented into individual lobes and surface sterilized using a mixture of H_2O_2 (30 %) and absolute ethanol in the ratio of 1:1 v/v through vortexing the above mentioned solution for five times. The surface sterilized lobes were then transferred into a sterile petridishes and crushed using a glass rod in distilled water. The crushed nodules were then streaked in a *Frankia* defined minimal medium and incubated at 28±2^o C for two-three months. The BAP medium was used for inocula preparation for further study.

2.3 Isolation and quantification of AM spores

Isolation of AM spores was done by using wet sieving and decanting technique of Gerdemann and Nicolson [5] and Singh and Tiwari [6]. 50gm of soil were suspended in 500 ml water and decanted on a series of sieves. Spores retained on the mesh were recovered by wash and then transferred to the petridish and counted under stereo-binocular microscope. The isolated spores were identified by using the keys of Trappe [7], Walker [8], Schenck and Perez [9], Morton and Benny [10] and Mukerji [11] and Enet websites such as www.mycorrhiza.com,www.ffp.csiro.aug.utk/research/mycorrhiza/intro&http://zor.zut.edu.pl/Glomeromycota/index.html

2.4 Mycorrhizal quantification

For quantitative estimation of VAM spores Gaur and Adholeya's [12]) modified method was used. The filter paper was divided into many small sectors and the total numbers of spores counted by adding the number of spores present in each sector under stereo-binocular microscope.

2.5 Colonization of VA Mycorrhizae

It was studied by rapid clearing and staining method of Phillips and Hayman [13]. The cleaned root segments were cut into 1cm each and placed in 10% KOH solution for 24 hours at room temperature. KOH was decanted, washed with water and then the root segments were acidified with 1% HCl for 3-5 minutes. The roots were then submerged in 0.5%, Trypan blue for 24 hours. After 24 hours, the segments were destained with Lactophenol and then observed using lactic acid: glycerol (1:1) solution for mounting.

3. Results and Discussion

The target plant species was screened for rhizospheric microbial association and it was found that this plant species were having nodules in roots (Plate 2) and nodular micro-endosymbiont and was found to be in a symbiotic relationship with the actinorhizal nitrogen fixing bacteria, *Frankia* sp. *Frankia* are filamentous bacteria, forming nodule like symbiotic associations with the roots of higher several plant species collectively known as "Actinorhizal Plant' [14]. These are gram positive bacteria that are known to nodulate about eight plant families representing 25 genera of woody, dicotyledonous, perennial angiosperms [1]. The families containing actinorhizal plant species are Betulaceae, Casuarinaceae, Myricaceae, Rosaceae, Rhamnaceae, Elaeagnaceae, Datiscaceae and Coriariaceae.

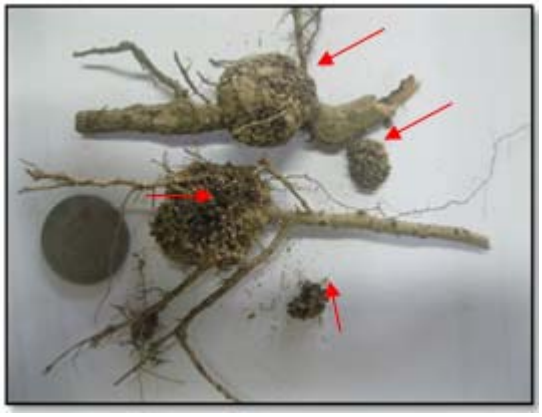

Plate 2: Root nodules (arrows show nodule aggregates) of *E. latifolia* inhabited by *Frankia* sp.

The nodules of *Elaeagnus latifolia* consisted of numerous nodule lobes that did not bear nodule roots. The number of nodule lobes was related to the age of the nodules with older nodules having more nodule lobes than young, developing nodules. Newcomb et al. [15] reported the root nodules of *E. umbellata* consisted of numerous lobes. The nodules in *E. latifolia* were globular in shape and mostly found in clusters of more than two or three (Plate 2).

The transverse section of the root nodule of *E. latifolia* revealed the presence of a single layered epidermis and isodiametric cortical cells interspersed with dark stained infected cortical cells which were comparatively larger in size as compared to that of the neighbouring cells. Nodule sections stained with saffranin showed dark pink colour indicating the actinomycete hyphae of *Frankia* sp. infection in the nodule cells. Infected cells were generally larger than the un-infected cortical cells (Plate 3a) and one unidentified endophytic fungus in the central cavity of the nodule which is having septate darkly stained hyphae (Plate 3b). The bacterium is very slow growing and it appeared after two to three months on BAP medium in *in vitro* condition and further research on it is under process.

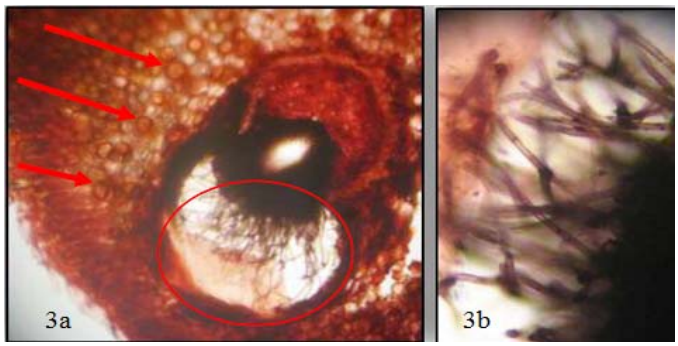

Plate 3a: T.S. of root nodule of *E. latifolia* showing bacterial colonies in nodular cortical cells (arrows) and endophytic fungus (in red circle); 3b: unknown endophytic fungus?

Transverse section of nodules obtained from the root of the target plant species revealed the presence of the massive proliferating bacterial hyphal system and large spherical vesicles of *Frankia* species (Plate 4, X 1000) at high magnification. Newcomb et al. [15] described similar pattern of arrangement of cells and tissues in the nodule lobes of *E. umbellata* to those patterns observed in other actinorhizal root nodules [15], [16], [17]. In *E. umbellata* a central vascular cylinder was surrounded by an inner cylinder of endodermis and an outer ring of cortical tissue, the middle layers of which were infected by the actinomycete endophyte. The nodule lobe meristem, which consisted of isodiametric cells containing many small vacuoles, was located at the distal end of the central vascular cylinder of each nodule lobe. Mitoses were observed only in the nodule lobe meristems. Endophytic hyphae grew towards the nodule lobe meristem and infected cells which were recently derived from this meristem. The endophytic actinomycete hyphae proliferated within the infected cells; the host cells grew and eventually formed large infected cells containing both filamentous hyphal and spherical vesicular forms of the endophyte. The outer covering of the nodule lobe consisted of either epidermal or phellogen cells, the vacuoles of which usually contained large phenolic deposits.

Plate 4: Bacterial actinomycete hyphae and vesicles of *Frankia* sp. within the cells of root nodule (in red circle)

After screening *Elaeagnus latifolia* for endomycorrhizal infections, it was found that all the three types of root infection/colonization namely hyphal, vesicular and arbuscular were observed in roots. Till dates no reports have been published in this aspect on the said species. There are few published reports on mycorrhizal associations with *E. angustifolia*. Khan [18]), investigated the incidence of mycorrhizae with 89 plant species from Pakistan, did not find any mycorrhizae in *E. angustifolia*. However, later on Riffle [19] showed that *E. angustifolia* seedlings were infected with vesicular-arbuscular mycorrhizae.

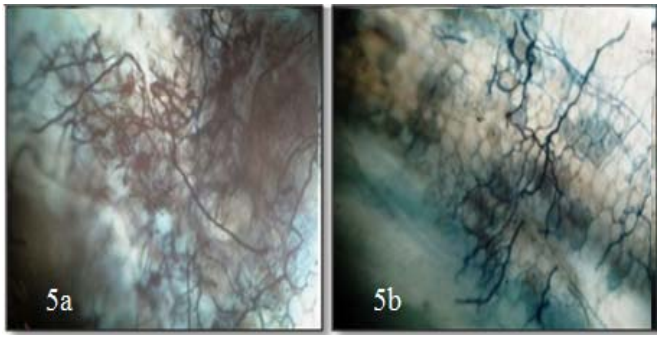

Plate 5a & 5b: Massive proliferating extraradical hyphae of endomycorrhizal fungi

Our study reveals that the roots had 90% hyphal infection, 70% arbuscular infection and 25% vesicular infection (Fig. 2). While, total mycorrhizal root colonization was present in all the roots (100 %). The hyphae were long and formed massive intracellular proliferating system (Plate 5a). They were of variable diameter and length (Plate 5b). The vesicles were oval to globose in shape, variable in size and extended intercellularly while the arbuscules were limited intracellularly (Plate 6). In *E. angustifolia* the percentage of root segments that contained vesicles, arbuscules or a combination of vesicles and arbuscles were 43, 9 and 25 respectively. The vesicles were thin walled, spherical to ovate, intercellular and occurred at a mean density of 21% of root while the arbuscles were intracellular and formed from the hyphae [19]. Smith and Smith [20] observed the mycorrhizal colonization of *Alnus acuminata* resembles the typical *Arum*-type in their entirely inter- and intracellular spread of the hyphae and vesicles, and the arbuscules were always simple and terminal. Similar results were observed by Maremmani et al. [21] in *Alnus glutinosa* (L.) Gaetrn. roots.

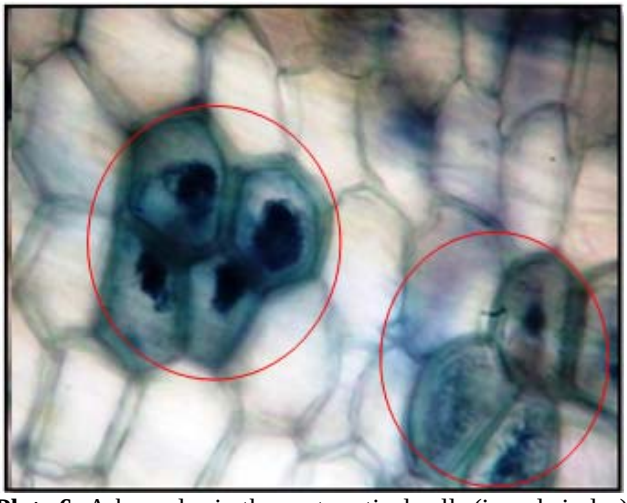

Plate 6: Arbuscules in the root cortical cells (in red circles)

Qualitative analysis revealed that the endomycorrhizae found in the rhizospheric soil of *E. latifolia* predominantly belongs to three genera viz. *Glomus*, *Acaulospora* and *Gigaspora* (Fig. 2). Altogether, 13 species of AM fungi were observed viz. *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe, *G. macrocarpum* Tulasne & Tulasne, *G. multicaule* Gerd. & Bakshi, *G. constrictum* Trappe, *Acaulospora laevis* Gerdemann & Trappe, *A. lacunosa* Morton, *A. apendicula* Spain, Sieverding & Schenck, *A. mellea* Spain & Schenck, *A.*

bireticulata Rothwell & Trappe, *Acaulospora* sp., *Glomus geosporum* Walker, *Gigaspora* species (Plate 7a,b).

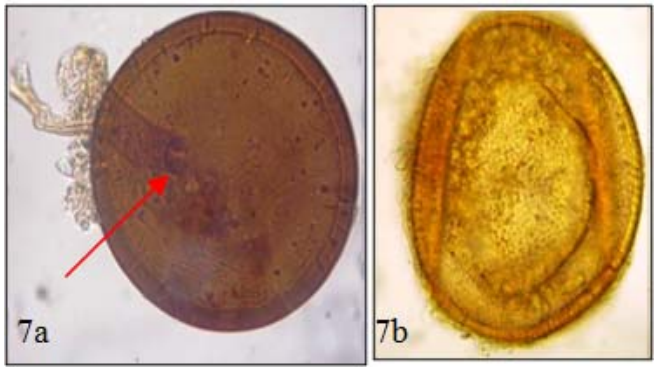

Plate 7a: *Acaulospora tuberculata* with soporiferous saccule and cicatrix (arrow) at 400X, 7b: *Acaulospora bireticulata* at X 400

The diversity of Arbuscular Mycorrhizae was retained by *Acaulospora* spp. (46.6%), followed by *Glomus* spp. (40%). While the occurrence frequency of *Gigaspora* spp. (13.3 %) was found to be the least among all the three genera (Fig. 3). Microscopic examination of stained root sections suggested the presence of AMF at all the sites where root colonization was observed while both *Glomus* and *Gigaspora* species occurred in root sections like a similar case of *Alnus glutinosa* [22] which is also an actinorhizal plant species.

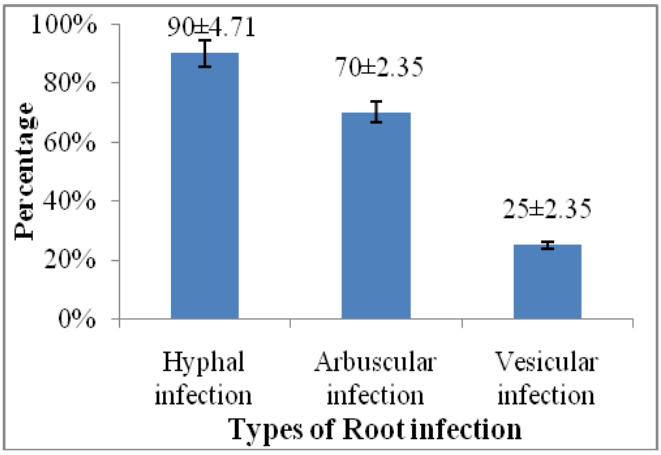

Figure 2: Percentage of different types of root infection in *Elaeagnus latifolia* L.

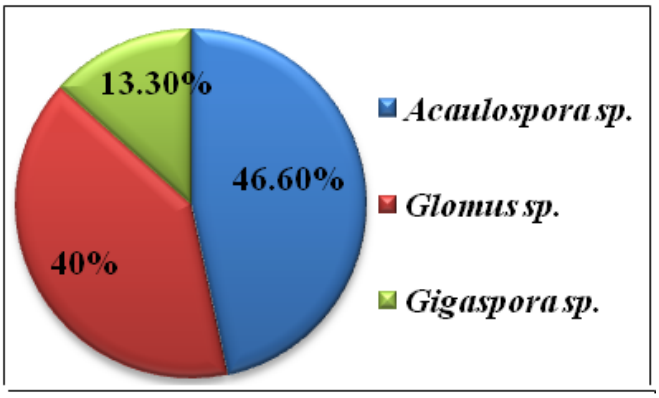

Figure 3: Generic diversity of Arbuscular Mycorrhizae associated with *Elaeagnus latifolia* L.

4. Conclusion

It is observed that the plant species harbours a number of mutualistic relationship with actinorhizal, endomycorrhizal and endophytic microbiota. Such parameters establish it as a model host for ecological succession. Due to its ability in fixing atmospheric nitrogen and phosphorus intake capacity *Elaeagnus latifolia* can also be used as a model system for agroforestry programmes such as in tea gardens etc. It can be speculated that such associations can play a vital role in bioactive compounds production too as it is an important medicinal plant species. There is a future scope of research on nodular micro-endosmybionts, host relationship and physiology in the target plant species along with isolation and culture of unknown endophytic fungus and their role in bioactive compounds production and the research on this aspect is under progress.

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Ms. Liza Handique is presently pursuing her Ph.D. research programme under the guidance and supervision of Dr. Vipin Parkash and doing research on AM fungi and their role in bioactive metabolite production through bio-inoculation.