Ectomycorrhizal and plant interaction on bioremediation of degraded land

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Extracellular enzymes degrade complex organic compounds into soluble substances and make them available for plants. These are important for nutrient cycling in the soil and ectomycorrhizal fungi (ECM) are an important source of these enzymes. In the present study, *Dipterocarpus retusus* seedlings native to Nagaland and Eastern Himalaya, India, were inoculated with *Scleroderma citrinum* and *Russula rosea* ECM. Soil enzymes like urease, dehydrogenase and nitrogen content were analysed in the rhizosphere region of the seedlings. *S. citrinum*-inoculated seedlings showed higher urease and dehydrogenase activity in rhizospheric soil and root surfaces and higher nitrogen content. Higher carbohydrate content was observed in *S. citrinum*-inoculated seedlings. Significant relation was found between ectomycorrhizal colonization and carbohydrate content. *S. citrinum* fungus was found to be a more effective symbiont with *D. retusus* seedlings during nursery practices for nutrient uptake in wasteland soil.

Keywords: Bioremediation, degraded land, extracellular enzymes, ectomycorrhizal fungi.

UNSUSTAINABLE land-use practices have put global land resources under severe threat. Therefore, restoring degraded lands is crucial to reclaim ecosystem services. Studies have shown that plants can be successfully used to remedy degraded lands. However, majority of the tree species that are considered the potential for bioremediation have a symbiotic relationship with ectomycorrhizal (ECM) or arbuscular mycorrhizal (AM) fungi[1]. More than 90% of land plants are associated with mycorrhizal fungi; two-thirds of them are arbuscular mycorrhizae, but the tree species predominant in temperate forests are ectomycorrhizal[2]. Mycorrhizal fungi play a key role in nutrient cycling and ecosystem functioning. ECM roots constitute an extensive portion of the nutrient-absorbing surface area in tree roots[3], and thereby also of the root system that can absorb and extract pollutants from the soil. ECM fungi have been shown to degrade a variety of organic pollutants. ECM fungi in the rhizosphere release extracellular enzymes to degrade organic matter present in the soil. The soil enzyme activity gives information on the nutrients present and the extent of degradation of organic matter as well as changes in soil quality. Analyses of enzymes in the soil provide knowledge of the impact of environmental changes or management. To understand the mechanism of nutrients uptake by ECM fungi, nutrient-assimilating enzymes in ECM mycelium have been studied[4,5]. Microorganisms are considered the primary source of enzymes in the soil, enzyme activities are strongly associated with microbial biomass. Soil enzyme activities are sensitive to both natural and anthropogenic disturbances, showing a quick response to the induced changes[6]. Urea is among the nitrogen fertilizers widely used in agriculture. Urease is the enzyme that catalyses the hydrolysis of urea to CO_2 and NH_3. ECM fungi posses the ability to enzymatically hydrolyse various compounds of plant and organic matter. The activity of urease and dehydrogenase is significant in nitrogen and carbon cycling in the ecosystem. Dehydrogenase enzyme is often used as a measure of any disruption caused by pesticides, trace elements or management practices to the soil microbial activity[7]. Dehydrogenase plays a significant role in the biological oxidation of soil organic matter by transferring hydrogen from the organic substrate to inorganic acceptors[8]. ECM fungi can take up organic nitrogen to be assimilated by the plants. Transfer of organic nitrogen by the mycorrhizal fungi, breaking down of organic nitrogen and the mechanism of its transfer across the fungal interface to the plant tissue help understand the extent of organic nitrogen used by the mycorrhizal fungi[9]. Generally, the mycorrhizal relationship is ignored as it occurs underground and is invisible, but mycorrhizal association plays a crucial and essential role in forest ecosystems. In the present study, enzymes released by the ECM fungi and nitrogen uptake from complex organic matter were monitored. We selected *Dipterocarpus retusus* Blume seedlings as they are listed as 'Vulnerable' on the *IUCN Red List*. *D. retusus* is native to Assam, Nagaland and Arunachal Pradesh in North East India. Two indigenous fungi, *Russula rosea* and *Scleroderma citrinum* were isolated as these are dominantly found in *Dipterocarpus* forests. The aim of this study was to assess the ectomycorrhizal colonization level of different fungi, seasonal variation of enzyme activity and to quantify total nitrogen uptake by the seedlings.

Materials and methods

Study site and establishment of seedlings

Seedlings of *D. retusus* were planted in the experimental field at the Nagaland University campus, Lumami (26°13.29′N and 94°28.430′E). Seedlings were planted at 1 m distance.

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They were allowed to establish for one month with weekly irrigation in the nursery before transplanting to the field. Seedlings were then inoculated with a fungal spore suspension. Twenty seedlings were maintained for each fungus and 20 as control.

Fungal isolation

Sporocarp of *R. rosea* and *S. citrinum* were collected from the forest of Lumami village, Nagaland between May and August and identified as outlined by Singer[10]. The young sporocarps were cultured on a modified Melin–Norkrans (MMN) medium[11]. Spore counting was done by serial dilution method[12]. Mycelium of the sporocarp was cultured in potato dextrose broth containing 4 g potato starch, 20 g glucose and 15 g agar. The volume was made up to 1000 ml with distilled water and autoclaved at 120°C for 15 min. Next, 1 ml of vegetative mycelial suspension was diluted in 9 ml distilled water (10^{-1}). From the previous dilution, 1 ml of sample was diluted again in 9 ml of distilled water and make the dilution factor to 10^{-2}, and so on up to 10^{-4}. Next, 1 ml of the suspension from each dilution was cultured in MMN and incubated at 25°C for five days. Spore counting was done as follows:

$$\text{Cells/ml} = \frac{\text{Average number of colonies}}{\text{Amount plated (ml)} \times \text{dilution factor}}.$$

Plant parameters and fungal assessment

Next, 2.2×10^5 and 2.7×10^5 spores/ml of *R. rosea* and *S. citrinum* were inoculated near the roots of seedlings of *D. retusus*. Twenty seedlings were maintained for each fungus and 20 were non-inoculated. Ectomycorrhizal colonized roots were harvested and the mycorrhizae colonized root tips were assessed and characterized on the basis of colour, branching shape and presence of emanating hyphae under a stereo microscope[13]. The control seedling roots were also harvested as the experiment was conducted under field conditions. ECM colonization/cm of total root length was calculated at the end of every fourth month, i.e. February–May, June–September and October–January. The ectomycorrhizal percentage was calculated according to Sharma[14] as follows:

$$\text{Percentage of ECM} = \frac{\text{Total number of dichotomous branched rootlets}}{\text{Total number of branched rootlets}} \times 100.$$

Enzymes were analysed during different seasons, i.e. winter (December–February), spring or pre-monsoon (March–May), summer or monsoon (June–August), and autumn or post-monsoon (September–November). The estimation of different enzymes was done using standard protocols.

Urease enzyme: Rhizospheric soil and seedling roots were analysed for urease activity during different seasons, i.e. summer, autumn, winter and spring[15]. Soil and root samples were crushed, passed through 2 mm mesh and sieved. Thereafter, they were stored at 4°C till further use. Then 1 g of sample was taken in a 100 ml volumetric flask, and 1 ml of toluene was added to it and allowed to stand for 15 min. Next, 10 ml of 750 mM sodium phosphate buffer solution (pH 7) and 5 ml of 10% urea were added and mixed well. The sample solution was incubated at 37°C for 3 h. After incubation, the flask was made up to 50 ml with distilled water and filtered through Whatman No. 42 filter paper. Then, to 1 ml of the filtrate, 3 ml of sodium hypochlorite solution (containing 0.9% active chloride) and 5 ml of phenolate solution were added. The sample was allowed to stand for 20 min and the final volume was made up to 50 ml with distilled water. The sample was read at 630 nm absorbance in a spectrophotometer. The ammonium (NH_4^+-N) content of the filtrate was estimated with reference to the calibration graph plotted. The urease activity was expressed as μg of N hydrolysed/g of dry sample per 3 h at 37°C. For each sample, a blank sample was run simultaneously where instead of 10% urea, 5 ml of distilled water was added.

Dehydrogenase enzyme: Root and rhizospheric soil were used to study for dehydrogenase activity during different seasons by the 2,3,5-triphenyl tetrazolium chloride (TTC) method[16].

Root and soil samples were ground and passed through a 2 mm mesh. Next, 1 g of sample was taken in a tube, to which 0.1 g of calcium carbonate and 1 ml of 1.5% of TTC were added, mixed thoroughly and plugged in with a rubber stopper. The sample was incubated at 30°C for 24 h. The control contained Tris buffer without TTC. After incubation, triphenyl formazan (TPF), a product from the reduction of TTC, was extracted by adding 10 ml of methanol to each tube and mixed well for 1 min. The aliquot was extracted by filtering through Whatman No. 42 filter paper. The absorbance of the red colour sample was read at 485 nm in the spectrophotometer. The enzyme activity was estimated by a standard graph plotted using the range of TPF. Enzyme activity was expressed as μg of TPF/g dry sample per 24 h.

Total nitrogen: Total nitrogen content of rhizospheric soil and root was determined using Kelplus Nitrogen Analyzer (Kes20LR AL and Kelvac AV). Root and soil samples were ground, sieved and passed through the 2 mm mesh. Next, 1 g of oven-dried sample, 10 ml of concentrated sulphuric acid and 3 g of catalyst (potassium : copper sulphate, 5 : 1) were mixed and loaded in the digestion unit. The sample was digested for 1½ h till it turned green in colour. Then 30 ml of distilled water was added to the sample. Boric acid (25 ml) with a few drops of mixed indicator was taken in a 250 ml conical flask that was placed at the receiver end. Next, 40 ml of 40% alkali (KOH) was added to the sample solution, followed by distillation for 9 min. During

the process, liquid ammonia was collected in the boric acid flask. When boric acid turned colourless, the flask containing ammonia was used for titration. The samples collected from distillation were titrated with 0.1 N hydrochloric acid. A blank sample was also used.

$$\text{Nitrogen } \% = \frac{14.01 \times 0.1\text{ N} \times (\text{TV} - \text{BV}) \times 100}{W \times 100},$$

where TV is the titrate value of the sample, BV the titrate value of the blank sample and W is the weight of the sample.

Statistical analyses

Paired t-test and correlation coefficient ($P \leq 0.5$) were calculated for carbohydrate content, enzymes and total nitrogen content using SPSS 17.0 software.

Results and discussion

Ectomycorrhizal colonization percentage to seedlings root of Dipterocarpus retusus

Colonization of ECM fungi to the seedling roots was found to vary during different months. After transplanting, the ectomycorrhizal colonization percentage was assessed in the sixth month. At the 12th and 20th months, colonization of *R. rosea* was found higher, but at the 6th, 8th and 24th month *S. citrinum* colonization was found higher after inoculation to the seedling roots (Figure 1). The variation in the colonization percentage of both fungi during different seasons could be due to fungi's adaptability to different climates and seasonal changes. This is supported by the study of Ekblad *et al.*[17], who observed that seasonal variation in ECM formation might be driven by abiotic variables, notably light, temperature and moisture and phonological phenomena both in the host and symbionts. The colonization percentage of both fungi inoculated at the 12th and 24th months during the dry season (i.e. winter season) was found to decrease. The control seedling roots were also found to be colonized by mycorrhizal fungi as the present study was conducted in open field conditions. The ectomycorrhizal fungi colonization percentage was significantly higher in inoculated seedlings than the control seedlings. This suggests that artificial inoculation of ECM fungi is beneficial for the seedlings, survivability where indigenous fungi find it difficult to associate with the exotic seedlings. Inoculation of specific mycobiont partner can facilitate the growth and establishment of plant species in degraded ecosystem.

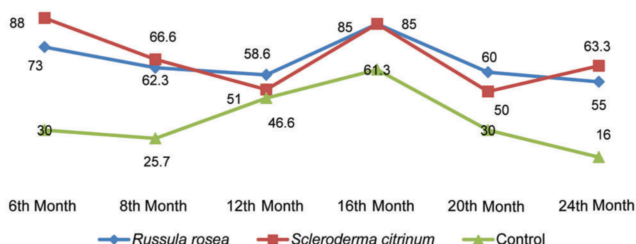

Figure 1. Ectomycorrhizal colonization percentage in seedlings of *Dipterocarpus retusus*. Error bar is the standard error of mean ($n = 5$).

Enzyme activity of the seedlings

Urease content in the rhizospheric soil of *S. citrinum*-inoculated seedlings was found to be higher during summer, autumn, winter and spring than the uninoculated and *R. rosea*-inoculated seedlings. A significant relationship was found between urease activity and ECM colonized roots (Figure 2). *R. rosea*-inoculated seedlings were found to be higher during the summer, winter and spring seasons. A significant relationship was found between fungal colonization and the urease activity of seedling roots (Figure 3). Variation in the enzyme activity in rhizospheric and seedlings roots by different fungi depends on the plant–microorganism interaction factor. Rhizospheric enzymes are controlled by several factors such as soil properties, plant roots and microorganism characteristics. In the rhizosphere, the biological activity of plant roots such as uptake, respiration and exudation affect the soil biochemical parameters[18]. The present study was conducted in open-field conditions. Therefore, besides the inoculated fungi, several other microorganisms might have affected the soil enzyme activity in the rhizosphere.

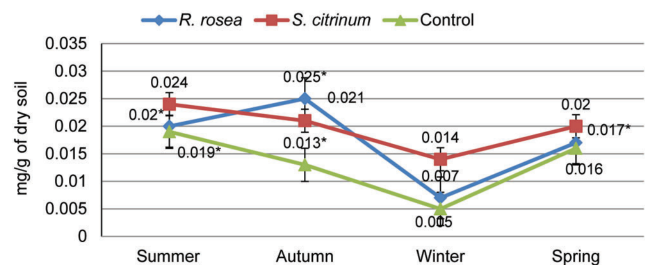

Figure 2. Urease activity in rhizospheric soil of *D. retusus* under different fungal treatments and control. Mean ± SE ($n = 3$), paired t-test, $^*P \leq 0.5$.

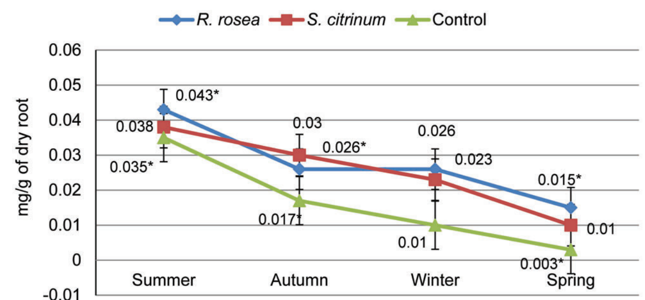

Figure 3. Urease content of *D. retusus* seedling roots under different fungal treatments and control. Mean ± SE ($n = 3$), paired t-test, $^*P \leq 0.5$.

The dehydrogenase activity of both fungal inoculated seedlings was found to be higher during summer. Dehydrogenase content in rhizospheric soil was higher for *S. citrinum* during summer and winter. However, during spring and autumn, *R. rosea*-inoculated seedlings were higher in the rhizospheric soil (Figure 4). Enzyme activity in the rhizospheric soil of both fungal inoculated seedlings was found to be higher compared to control seedlings. A significant relationship was found between ECM colonization and dehydrogenase activity (Figure 4). During different seasons, dehydrogenase activity was found to be higher for *S. citrinum*-inoculated seedlings roots compared to *R. rosea*-inoculated and control seedlings (Figure 5). A significant relationship was found between ECM colonization and the dehydrogenase content of the seedling roots (Figure 5). Dehydrogenase enzyme occurs as an intracellular enzyme in all living microbial cells and is used as an indicator of microbial activity[8]. *S. citrinum* was found to be an effective symbiont with *D. retusus* in nutrient degradation, as the dehydrogenase activity is directly associated with actively growing microorganisms.

Urease and dehydrogenase activity showed variation during different seasons. Summer and autumn were most favourable for enzyme activity and showed higher enzyme content. Kumar *et al.*[6] reported the highest dehydrogenase during the summer and autumn seasons, which was correlated to the microbial population in the soil. During the winter season, urease and dehydrogenase activity was less. This could be because ECM fungi live as saprotrophs when their hosts perform less photosynthetically. The reduced level of ECM colonization resulted in low enzyme activity during winter. The enzyme activity of ECM fungi depends on their trait and functions. Buee *et al.*[19] have hypothesized that ECM fungal species that colonize roots vary in their capacity to produce extracellular enzymes that target the carbon-rich substrate and nutrients to be broken down. Urease and dehydrogenase content of ECM-inoculated seedlings roots was found to be higher than in rhizospheric soil. Thus, ECM fungi contribute a considerable amount of enzymes to the microbial population in the soil. Release of enzymes through ECM roots into the rhizosphere may contribute to the degradation of nutrients from the soil, and cycling of nutrients and carbon in the ecosystem. Mamatha *et al.*[20] reported higher dehydrogenase activity in the soil covered by plants and in the rhizosphere than in non-rhizospheric soil. In the present study, in it is likely that the other indigenous ECM fungi present in the soil might have affected the enzyme and nutrient status of the rhizospheric soil.

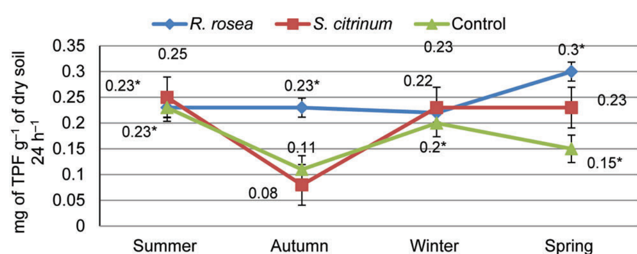

Figure 4. Dehydrogenase activity in rhizospheric soil of *D. retusus* under different fungal treatments and control. Mean ± SE ($n = 3$), paired *t*-test, $*P \leq 0.5$.

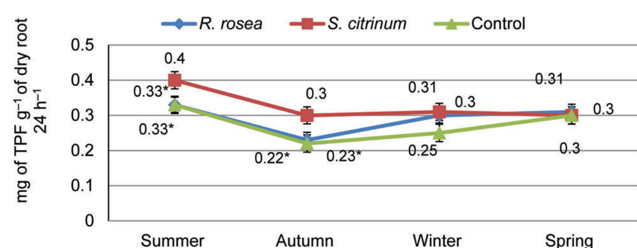

Figure 5. Dehydrogensae activity of seedling roots of *D. retusus* under different fungal treatments and control. Mean ± SE ($n = 3$), paired *t*-test, $*P \leq 0.5$.

Nutrient uptake of the seedlings

S. citrinum-inoculated seedling roots had higher contents of nitrogen compared to *R. rosea*-inoculated and control seedlings (Figure 6). In the rhizospheric soil, the nitrogen content of the inoculated and control seedlings did not show much variation (Figure 6). During spring, summer, autumn

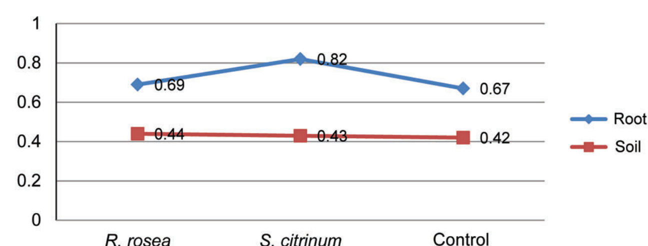

Figure 6. Total nitrogen content (%) in rhizospheric soil and seedlings of *D. retusus*. Error bar is the standard error of mean percentage ($n = 3$).

Table 1. Correlation between urease and nitrogen content in rhizospheric soil

Ectomycorrhizal (ECM) fungus	Spring (r)	Summer (r)	Autumn (r)	Winter (r)
Russula rosea	0.997*	0.928	1.00**	0.561
Scleroderma citrinum	0.999*	0.999*	0.988*	0.896
Control	0.795	0.999*	0.553	0.803

Pearson's correlation coefficient (r), significance level $*P < 0.05$, $**P < 0.01$.

Table 2. Correlation between urease and nitrogen content of seedlings

ECM fungus	Spring (r)	Summer (r)	Autumn (r)	Winter (r)
R. rosea	0.988*	0.990*	1.00**	1.00**
S. citrinum	0.993*	1	0.993*	0.988*
Control	0.955	0.996*	0.994*	1.00**

Pearson's correlation coefficient (r), significance level $*P < 0.05$, $**P < 0.01$. Error bar is the standard error of mean ($n = 3$).

seasons significant correlation was between urease enzyme activity and nitrogen content in rhizospheric soil of both the fungal inoculated seedlings (Table 1). This suggests that the urease enzyme plays a vital role in the assimilation of soil nitrogen. Plants in both temperate and tropical forest preferred to take up ammonia, with organic nitrogen as the second most needed nitrogen source[21]. *S. citrinum* was found to be effective in improving the nitrogen requirement of the seedlings. A significant relationship was found between urease enzyme activity of seedling roots and nitrogen content (Table 2). Nitrogen uptake of fungal-inoculated seedling roots is proportional to the release of the urease enzyme by ECM roots, which helps nitrogen metabolism providing biologically major source for nitrogen. Read[22] suggested that ammonium is the major source of nitrogen, and ECM fungi preferentially use the ammonium ion when grown in culture.

Conclusion

The ability of ECM fungi to metabolize a variety of complex organic matter is essential in various soil processes such as nutrient cycling. ECM colonization level was found to depend on seasonal variation. ECM colonization percentage was higher during the 6th, 8th, 16th and 20th months. Colonization was higher between May and September, i.e. during the summer and autumn seasons. ECM colonization is not a concurrent process; it is affected by different abiotic factors such as soil pH and moisture. *S. citrinum* was found to be a better symbiont with the host plant in improving nitrogen status in the plant by increasing the enzyme activity. Level of ectomycorrhizal colonization percentage with the host plant, mycorrhizae growth and ectomycorrhizal fungal interaction with the host might affect the nutrient uptake by different ectomycorrhizal fungal species. Therefore, it is necessary to assess the role of different ectomycorrhizal fungal species under different environmental conditions. In the present study, *S. citrinum* inoculated seedlings showed higher dehydrogenase enzyme content in seedlings roots and in rhizospheric soil. This shows that *S. citrinum* plays an important role in biochemical functions. The total nitrogen content of *S. citrinum*-inoculated seedling roots was higher than that the control and *R. rosea*-inoculated seedlings. Inoculation of ECM fungi is important in nutrient-poor and acidic soils to improve nutrient uptake by the plants. Nitrogen uptake depends on resource availability, degradation of organic matter in the soil and the type of fungal hyphae exploration in the soil. Enzyme activity was found to be influenced by seasonal variation. Variation in the enzyme activity of ECM species during different seasons could be due to the competitive ability of some ECM fungi, where one species became the primary symbiont and the other secondary. This variability in the function of dominant individual species also depends on the environmental conditions and physiology of the host. ECM species also act as a biological indicator of the soil as their growth and biomass is influenced by the nutrients present in the soil. It is necessary to determine the various physiological abilities of ECM species with different hosts to increase ecosystem productivity.

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