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Nutrient status of rhizosphere soil and *Pinus gerardiana* seedlings inoculated with *Scleroderma polyrhizum*

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ABSTRACT

Pinus gerardiana is a highly valuable conifer species of dry temperate, semi-arid and arid zones of the northwestern Himalayan region. The large scale harvesting of cones for nuts along with extreme weather conditions result in poor natural regeneration of this species. Thus this endemic species is facing risk of extinction in prospective times and needs special attention for conservation. In the present study, planting stock of *P gerardiana* was produced by artificial inoculation of *Scleroderma polyrhizum* and the nutrient status of rhizosphere soil and seedlings was investigated just before planting. The content of macro and micronutrients in the roots and shoots of artificially inoculated seedlings was found to be higher than in the control. The roots had higher total carbon, nitrogen and phosphorus content, whereas, the shoot samples had higher calcium, magnesium, manganese, copper and iron content. The study of corresponding seedling rhizosphere soil demonstrated a higher concentration of nutritional components in the rhizosphere soil of inoculated seedlings compared to control. Aside from that, the inoculated seedlings' rhizosphere soil was more acidic than the control. The electrical conductivity of inoculated seedlings was also higher in the rhizosphere soil. Thus the study revealed that artificial inoculation of *P gerardiana* with *S polyrhizum* enhances the nutrient absorption in the seedlings.

Keywords: *Pinus gerardiana*; ectomycorrhiza; *Scleroderma polyrhizum*; nutrient uptake

INTRODUCTION

Pinus gerardiana Wall ex D Don is an important gymnosperm species in the northwestern Himalaya. The multipurpose coniferous species was discovered by Captain Patrick Gerard and the scientific name *Pinus gerardiana* attributes to the name of its discoverer (Kumar et al 2017, Singh et al 2021). It is a member of the Pinaceae family and is also known as Chilgoza or Neoza pine. It prefers dry temperate climate and thrives best in slightly acidic to neutral soil (Akbar et al 2014). The species is appropriately referred to as the Champion of the Rocky Mountains since it inhabits and thrives in the challenging habitats and stress areas of the Himalayan region (Malik et al 2012, Singh et al 2021). *P gerardiana* grows between 1,800 and 3600 metres amsl and is distributed in Afghanistan, Pakistan with scattered appearance in northwestern Himalayas. Pure chilgoza pine forests can be found in Himachal Pradesh in Pavari, Baspa, Karchcham and Akpa regions of Kinnaur district and in minor patches in Pangi and Bharmour areas of Chamba district of Himachal Pradesh. The species has been recorded from Kishtwar and Dachhan regions in Jammu and Kashmir. *P gerardiana* produces cones containing edible nuts high in linoleic acid, unsaturated fatty acids, oleic acid, tocopherol, thiamine, riboflavin, pantothenic acid and minerals such as phosphorus, magnesium, calcium, potassium, manganese, iron and zinc (Haq et al 2013, Cai et al 2017, Rehman et al 2017). The trading of nuts generates significant returns; thus the cones are harvested in large quantities to extract the nuts (Shalizi and Khurram 2016). According to the International Union for Conservation of Nature (IUCN) red list category, the species is listed as near threatened because its natural habitat has declined significantly due to unscientific and ruthless harvesting (https://www.iucnredlist.org/species/ 34189/2850009). In this context, this ecologically and commercially important tree species is under tremendous biotic and abiotic stress and proceeding towards extinction (Kumar et al 2017). Over-harvesting, poor survival and establishment of seedlings and harsh environmental conditions are the key causes of the shrinking of Chilgoza forest cover which is diminishing and depleting rapidly. Therefore, production of seedlings with quality growth characters and ability to survive on planting are the need of the hour.

In nature, higher plants exist in close association with lower organisms including mycorrhizal fungi. Mycorrhizae are mutually beneficial associations between plant roots and fungi. This symbiotic interaction between soil fungi and plant roots is widespread and exists in majority of terrestrial plants (Smith and Read 2008, Brundrett and Tedersoo 2018). The roots of the majority of conifers are colonised by ectomycorrhizal fungi which provide a variety of benefits to the host plants (Rapparini and Penuelas 2014, Singh et al 2020). These symbionts play an important role in seedling establishment and survival by influencing growth and nutrient cycling (Phillips et al 2013, Mrak et al 2017, Sebastiana et al 2017). The majority of ectomycorrhizal fungi are basidiomycetes and ascomycetes and they are known to improve the process of soil nutrient mobilisation, absorption and translocation to the roots (Dominguez-Nunez and Albanesi 2019).

The ectomycorrhizal fungi have the potential to uptake the various macro and micronutrients including phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), copper (Cu), manganese (Mn) etc from the soil and provide these nutrients to the host plants (Becquer et al 2019). Apart from this, the ectomycorrhizal fungi also help in faster decomposition of organic complexes and promote the process of soil N cycling and soil C sequestration (Orwin et al 2011, Cairney 2012, Clemmensen et al 2014, Bodeker et al 2014, Lindahl and Tunlid 2015). Appreciating the fundamental role of ectomycorrhizal fungi in various soil processes, nutrient dynamics and forest productivity, there is increasing need to incorporate the mycorrhizal fungi with the seedlings of forest trees and identify their role in seedling growth and nutrient uptake (Brzostek et al 2014, Tapwal et al 2015, Sulman et al 2017). Artificial inoculation of conifer with mycorrhizal fungi in nursery can play an important role towards better growth and establishment of its seedlings under the field conditions. The study was conducted in Kinnaur district of Himachal Pradesh to investigate the status of major nutrients in the *P gerardiana* seedlings artificially inoculated with mycorrhizal fungi.

MATERIAL and METHODS

Study area: The research work was carried out at the nursery of the Himachal Pradesh State Forest Department at Akpa in Kinnaur district, Himachal Pradesh, the natural zone of *P gerardiana*. The climate of the area is characterised by temperature extremes, severe dryness and extremely evaporative wind. During protracted winters, which normally begin at the end of September, the area receives heavy to moderate snowfall that lasts until May and, therefore, snow is the only form of precipitation.

Culturing of mycobiont and artificial inoculation: The sporocarps of *Scleroderma polyrhizum* (JF Gmel) Pers, which were closely associated with *P gerardiana*, were collected and pure culture was isolated on potato dextrose agar (PDA) following tissue culture technique. To do this, the sporocarp was surface-sterilised with alcohol, an incision on the pielus was made with a sterilised blade and a little tissue was picked with forceps to inoculate on PDA. By consecutive sub-culturing, pure culture was obtained. Following Stoller (1962), mass inoculum was produced on wheat grains in polypropylene bags (150-200 g/bag).

The seeds were collected from healthy trees and sown in polybags (18" x 9") that had previously been filled with fumigated soil. The mycorrhizal inoculum containing *S polyrhizum* produced on wheat grains was mixed with the top 1/3 of soil. The uninoculated polybags served as control. After spawning the soil, surface-sterilised seeds were sown in each bag.

Estimation for nutrient status of seedling and rhizosphere soil: The elemental composition of the 21-month-old seedlings and rhizosphere soil was estimated just before outplanting. The elemental composition of roots and shoots was analysed separately by following standard methodology. The organic carbon was determined following the rapid titration method of Walkley and Black (1934), nitrogen using the Kel-Plus nitrogen estimation system (Model: Classic-DX), phosphorus following the stannous chloride method (Bray and Kurtz 1945) using a UV-Vis spectrophotometer (Model: Evolution 2001) and potassium was estimated using a flame photometer (Model: ESICO:1385). The micronutrients Cu, Zn, Fe, and Mn were measured using atomic absorption spectrometer (Model: AAS-7000). In addition to the elemental composition, the rhizosphere soils of the corresponding seedlings were analysed for estimation of the soil reaction and electrical conductivity (1:2.5 soil water suspension) using digital pH metre (ESICO: 1012) and electrical conductivity metre (ESCIO: 1601)

RESULTS and DISCUSSION

The diversity of *P gerardiana* ectomycorrhizal associates in the NW Himalaya has been investigated (Tapwal et al 2021). The major mycorrhizal associate was identified as *S polyrhizum* (Plates 1a-b). The culture of *S polyrhizum* was isolated from sporocarp and subsequently maintained on PDA (Plate 1c). Mass inoculum was produced on wheat grains (Plate 1d) and transported to the experimental site Akpa, district Kinnaur, Himachal Pradesh for artificial inoculation of seedlings. In previous study, the enhancement in the growth metrics of the artificially inoculated seedlings has been recorded (Tapwal et al 2022). One of the reasons for the rise in growth characteristics of artificially inoculated seedlings (Plates 1e-f) could have been increased nutrient acquisition. Mycorrhizal roots increase the surface area of the plant root system increasing the contact area around the roots for improved macro and micronutrient uptake (Becquer et al 2019).

The mycorrhizal symbionts promote the efficient use of soil nutrients by host plants. The positive mycorrhizal growth response triggers the increase in the biomass and disease tolerance of host seedlings (Bernaola et al 2018, Zhang et al 2019). The increase in biomass may be due to the increase in concentration of nutrients in the seedlings (Balliu et al 2015).

Ectomycorrhizal fungal associations are definitely advantageous to host plant and enhance growth and increase nutrient uptake and also inhibit stress (Dowarah et al 2022). In the present study, the nursery stock of *P gerardiana* was produced through artificial inoculation with *S polyrhizum*, as stated in the protocol. The elemental makeup of the shoots and roots of 21-month-old seedlings as well as the corresponding rhizosphere soil was determined just before outplanting. The results revealed that elemental composition in the shoots of control and inoculated seedlings was as follows: total carbon (52.30 and 56.42%); total nitrogen (2.10 and 2.62%), phosphorus (1.05 and 2.81%), potassium (12.50 and 14.81%), calcium (6.12 and 8.65 ppm), magnesium (59.18 and 55.41 ppm), manganese (9.87 and 10.87 ppm), copper (12.05 and 9.08 ppm), zinc (12.69 and 16.05 ppm) and iron (61.45 and 89.62 ppm) respectively. The composition of tested elements in roots of same seedlings for control and inoculated one were found to be as total carbon (54.23 and 62.14%), total nitrogen (3.11 and 4.23%), phosphorus (1.26 and 3.21%), potassium (14.52 and 16.42%), calcium (7.04 and 8.27 ppm), magnesium (31.25 and 36.47 ppm), manganese (5.21 and 6.35 ppm), copper (7.12 and 8.26 ppm), zinc (8.47 and 10.29 ppm) and iron (61.32 and 79.02 ppm) respectively. The data analysis revealed that concentration of macro and micronutrients in the roots and shoots of artificially inoculated seedlings was higher than the control. Total carbon, nitrogen and phosphorus content was higher in the roots, while Ca, Mg, Mn, Cu and Fe content ws higher in the shoot samples (Table 1).

The underground mycelial network of ectomycorrhizal fungi promotes the turnover of soil organic matter and cycling of various nutrients in forest ecosystems (Frey 2019). Ectomycorrhizal symbiotic associations in coniferous species improve the uptake of inorganic nitrogen (Baskaran et al 2017). Ectomycorrhizal fungi play a significant role in acquisition of macronutrients (N, P, K) and secondary nutrients like Ca, Mg, etc by the host plants (Neba et al 2016, Schmalenberger et al 2015). The plants can absorb nutrients from the soil by root hair or through mycorrhizae, which involves the nutrient acquisition via fungi. Arteaga-Leon et al (2018) inoculated *Pinus ayacahuite* seedlings with edible ectomycorrhizal mushrooms (*Helvella* cf *lacunosa* and *Hebeloma mesophaeum*) and found that the inoculated seedlings grew faster and had greater levels of major macro and micronutrients. Almost similar results were obtained in the present investigations.

The pH, electrical conductivity and status of the important major and minor nutrients were also determined in the rhizosphere soil of control and inoculated seedlings. The data revealed that the inoculated seedlings' rhizosphere soil was more acidic (pH 4.68) than the control (pH 5.22). The electrical conductivity of inoculated plants' rhizosphere soil was likewise higher (21.26 mS) than the control (19.23 mS). The elemental composition in the rhizosphere soil of control and inoculated seedlings was recorded as follows:

Table 1. Elemental composition of containerised seedlings

Element	Concentration			
	Root		Shoot	
	Control	Inoculated	Control	Inoculated
Carbon (%)	54.23	62.14	52.30	56.42
Potassium (%)	14.52	16.42	12.50	14.81
Nitrogen (%)	3.11	4.23	2.10	2.62
Phosphorus (%)	1.26	3.21	1.05	2.81
Ca (ppm)	7.04	8.27	6.12	8.65
Mg (ppm)	31.25	36.47	59.18	55.41
Mn (ppm)	5.21	6.35	9.87	10.87
Cu (ppm)	7.12	8.26	12.05	9.08
Zn (ppm)	8.47	10.29	12.69	16.05
Fe (ppm)	61.32	79.02	61.45	89.62

Table 2. Elemental composition of rhizosphere soil

Element	Concentration	
	Control	Inoculated
Carbon (%)	0.96	1.86
Potassium (kg/ha)	97.22	98.12
Nitrogen (kg/ha)	287.21	291.10
Phosphorus (kg/ha)	9.11	11.64
Ca (ppm)	10.26	12.22
Mg (ppm)	21.52	23.11
Mn (ppm)	0.54	0.68
Cu (ppm)	1.14	1.02
Zn (ppm)	0.98	0.81
Fe (ppm)	31.92	31.47

organic carbon (0.96 and 1.86%), potassium (97.22 and 98.12 kg/ha), nitrogen (287.21 and 291.10 kg/ha), calcium (10.26 and 12.22 ppm), magnesium (21.52 and 23.11 ppm), manganese (0.54 and 0.68 ppm), copper (1.14 and 1.02 ppm), zinc (0.98 and 0.81 ppm) and iron (31.92 and 31.47 ppm) respectively (Table 2). The concentration of each tested element was higher in the rhizosphere soil of inoculated seedlings in comparison to control seedlings. The rhizosphere represents the most complex habitat consisting of roots, soil and diversity of microorganisms including mycorrhizal fungi. This narrow zone in surrounding plant roots is home to a range of microbial communities that conduct a number of functions and influence plant growth (Mcnear 2013, Ahkami et al 2017). Arteaga-Leon et al (2018) inoculated the seedlings of *Pinus ayacahuite* with ectomycorrhizal fungi and recorded higher growth and major macro and micronutrients in the inoculated seedlings. The extra-matrical mycelium of ectomycorrhizal fungi has been reported to influence physical fragmentation and soil properties (Bonneville et al 2009).

CONCLUSION

P gerardiana is very important tree species of Kinnaur district of northwestern Himalayas and contributes a lot to the ecology and economy of the area. The unscientific harvesting and harsh environmental conditions result in its poor natural regeneration. Therefore, considerable efforts and combination of strategies like suitable harvesting method, appropriate plantation site selection and pre-conditioned quality seedlings are required for the conservation and planting success of the species. Ectomycorrhizal symbiosis is an effective biological mechanism for effective nutrient uptake and better seedling survival in nutrient-deficient areas. It is well

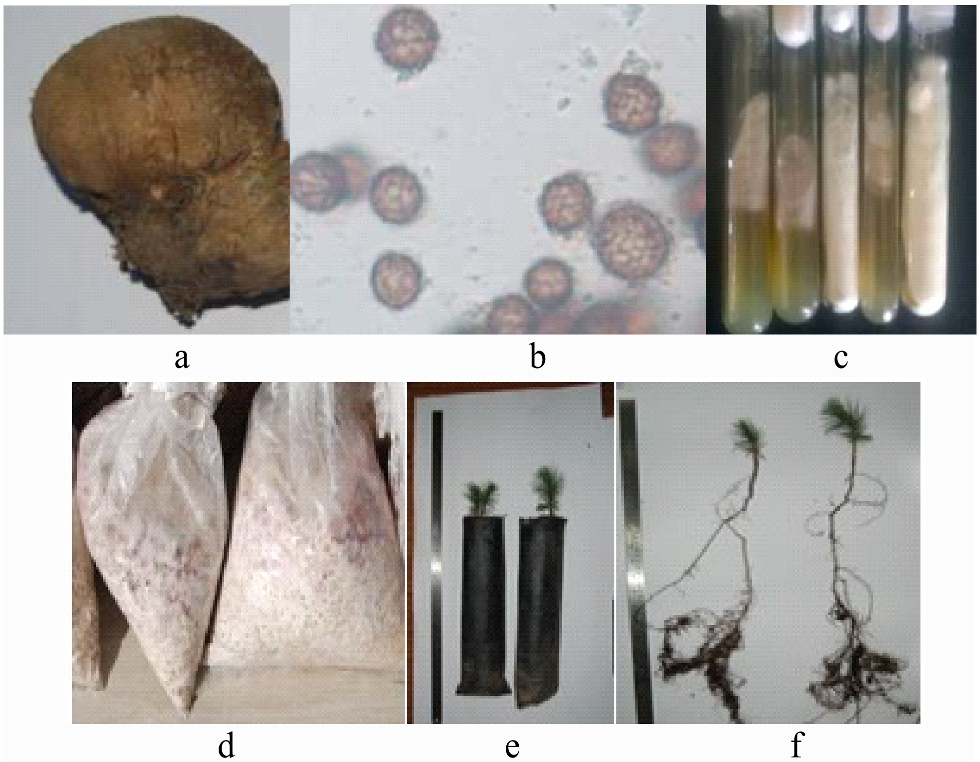

Plate 1. a) Sporocarp of *S polyrhizum*, b) Spores of *S polyrhizum*, c) Pure culture of *S polyrhizum*, d) Mass culture of *S polyrhizum*, e-f) *P gerardiana* seedlings

known fact that in vitro mycorrhizal inoculation of seedlings with suitable mycorrhizal fungi can reduce the nursery period and also help the seedlings establishment upon outplanting. The results of the study revealed that the seedlings of *P gerardiana* inoculated with the culture of *S polyrhizum* increased the absorption of nutrients and nutrition also improved the status of mineral content in above and below ground plant parts. Thus pre-conditioning of *P gerardiana* seedlings is important for better uptake of nutrients and finally it leads to optimal establishment and survival of seedlings in field conditions. The study indicated the host-fungus relationship and demonstrated the potential role of particular *S polyrhizum* – the fungal symbiont in restoration of *P gerardiana* forests.

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