General microflora, arbuscular mycorrhizal colonization and occurrence of endophytes in the rhizosphere of two age groups of *Ginkgo biloba* L. of Indian Central Himalaya

Ajay Kumar · Shipra Singh · Anita Pandey

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Abstract The populations of the general microflora (bacteria, actinomycetes and fungi) in the rhizosphere and their corresponding non-rhizosphere soil samples of *Ginkgo biloba* L. of two age groups (Group A, <25 years-young trees; Group B, >60 years-old trees) growing under a temperate location of Indian Himalayan Region (IHR) have been determined. Observations were also made for the diversity, distribution and colonization of arbuscular mycorrhizal (AM) fungi and occurrence of endophytes in roots of *G. biloba*. The population of general microflora was found to be higher in the rhizosphere of Group B trees, more clearly reflected in terms of rhizosphere: soil (R: S) ratios. Contrary to this, per cent colonization and spore densities of AM fungi were higher in the rhizosphere of Group A trees as compared to the rhizosphere of Group B. AM fungal colonization was observed mostly in form of loose coils. All the spores detected, belonged to the genus *Glomus* with five different types. Presence of endophytes (both bacteria and fungi) was observed in the cortical cells of *G. biloba* roots, more profound in case of Group B trees. Data suggest that, while the species of *Glomus* dominated the rhizosphere of

G. biloba, an inverse correlation exist between the colonization of general microflora and the colonization of AM fungi including endophytes.

Keywords *Ginkgo biloba* · Rhizosphere microflora · Rhizosphere effect · AM fungi · Endophytes

Introduction

The rhizosphere is a dynamic environment in which bacteria, viruses, fungi, and other microfauna develop, interact with each other, and take advantage of organic matter released by the roots. The diversity of rhizosphere microflora changes from one site to another and depends on the physio-chemical and biological properties of the soil. The ‘rhizosphere effect’ is defined as the overall positive influence of interactions between roots and the rhizoflora on the development of the plants. The symbiotic arbuscular mycorrhizal (AM) fungi develop an extensive hyphal network and provide water and nutrients to plants. Soil microorganisms can influence the soil structure and play important role for AM fungal colonization in roots [1, 2, 3].

Ginkgo biloba L., often referred as the only living member of the *Ginkgo* family (Ginkgoaceae), is recognized for its sturdiness, resistance to diseases and remarkable longevity. The species is interesting from the taxonomic and evolutionary point of view, as it is the only surviving genus and species of the Ginkgoales which reached its height in the Jurassic period. For centuries, it was thought to be extinct in the wild, but it was known to grow wild in at least two small areas in Eastern China in the Tian Mu Shan Reserve

A. Kumar · S. Singh · A. Pandey (✉)
Microbiology Lab,
Biodiversity Conservation and Management and
Biotechnological Applications Core,
G.B. Pant Institute of Himalayan Environment and
Development, Kosi-Katarmal Almora, 263 643
Uttarakhand, India
E-mail: anita@gbpihed.nic.in

Table 1 Description of the study sites

Sites	Longitude	Latitude	Altitude (amsl)	Rainfall (mm) per annum	Avg. temperature (°C)
Nainital	N 29°22'33.7"	E 079°27'36.3"	2078 m	4961	15.4
Chaubatia	N 29°37'17.8"	E 079°27'35.5"	1982 m	1190	16.8
Kalika	N 29°39'54.7"	E 079°28'43.7"	1742 m	1190	16.8

(<http://en.wikipedia.org/wiki/Ginkgo>). *G. biloba* is also growing at few places in protected areas of Indian Himalayan Region (IHR) and efforts are being made to propagate this plant mainly due to its importance in medicine. The leaf extract of the *G. biloba* contains pharmaceutically important flavonoides, glycosides and ginkgolides which improves blood flow, acts as antioxidant and mainly used as memory enhancer and anti-vertigo agent (www.uspharmacist.com). The objective of the present study was to acquire an insight into the microflora including bacteria, fungi and actinomycetes with special emphasis on AM colonization in the rhizosphere of *G. biloba* growing in temperate climatic conditions of IHR. Observations recorded on occurrence of endophytes are also described.

Materials and methods

Study site

All *G. biloba* plants, selected for this study, were growing in protected areas (Nainital: Distt. Nainital, Chaubatia: Distt. Almora and Kalika: Distt. Almora) of Kumaun region of Uttarakhand in IHR. Six different trees were selected and grouped into two major categories, each with three trees; Group A with younger trees (<25 years) and Group B with older trees (>60 years). While few old trees (Group B) are available in this region, young trees (Group A) have been raised using cuttings of the old trees. A brief description of the study sites is given in Table 1.

Sample collection

Rhizosphere and non-rhizosphere soil and root samples were collected in triplicates from each tree, at a depth of 10–15 cm after removing the top layers of humus and other vegetation. Soil samples were brought to laboratory in labeled polyethylene bags, sieved through 2 mm sieve, air dried, and subsequently stored at 4°C prior to further analysis.

Chemical analysis of soil

Soil samples in triplicates for each tree were analyzed for carbon and phosphorus content. Carbon content was de-

termined by wet-oxidation method and phosphorus content by using a spectrophotometer (Uvikon 931, Italy) following standard procedures [4].

Enumeration of general microflora

For enumeration of the general microflora, counts of bacteria, actinomycetes and fungi, were estimated on the basis of serial 10-fold dilutions following pour-plate method and taking appropriate dilution [5]. Trypton yeast extract agar, actinomycetes isolation agar and potato dextrose agar were used for estimation of bacteria, actinomycetes and fungi, respectively (all from Hi-media, Bombay, India). The plates were incubated at 25°C ± 2 for one week and the average number of colony forming units (CFU) per g oven dry weight of soil was calculated using the formula:

$$\text{CFU} = \frac{\text{Counts on the culture plate} \times \text{fresh weight of soil}}{\text{Weight of oven-dried soil}}$$

A numerical value for the rhizosphere: soil (R:S) ratio, representing microbial counts in the rhizosphere divided by the microbial counts in the non-rhizosphere soil was used as an index of the rhizosphere effect on the microbial population.

Root colonization by AM fungi

Root samples in triplicates for each tree were washed gently to remove adhering soil particles in running tap water. Fine feeder roots were cut into small segments (approximately 1 cm) and cleared in 10% KOH (wv⁻¹; 90° C; 1h), bleached with alkaline H₂O₂ (15 min), acidified with 1% HCl (vv⁻¹, approx. 10 min) and then stained with 0.05% Trypan blue (wv⁻¹, 90°C for 30 min) according to Phillips and Hayman (1970) [6]. Root segments were mounted on glass slide with lactophenol and observed under compound microscope (Nikon-Optiphot-2). A minimum of 100 segments for each sample were observed for the assessment of per cent colonization of AM fungi using the following formula:

$$\% \text{ AM colonization} = \frac{\text{Total number of root segments colonized}}{\text{Total number of root segments examined}} \times 100$$

Spore isolation and identification

Spores of AM fungi were isolated from rhizosphere soil by the wet sieving and decanting technique [7]. Approximately 25 g of individual air-dried rhizosphere soil sample was dispersed in 1 L of water in a beaker and the suspension was left undisturbed for 15–20 min. The suspension was then decanted through the stack of sieves 425 μm –250 μm –125 μm –40 μm (arranged in decreasing order of mesh size from top to bottom). Same process was repeated 2–3 times and the residue from each sieve was collected into petri plates with little distilled water. Intact AM fungal spores were examined and counted under stereomicroscope (Olympus OIC 1629) and identifications were made by observing diagnostic characteristics such as spore wall, colour, size and type of hyphal attachment according to Schenck and Perez (1990) [8] under compound microscope (Nikon-Optiphot-2).

Observations on endophytes

Transverse sections of roots were stained with lactophenol cotton blue by putting a drop over root sections. Stained sections were then mounted with lactophenol over glass slides and observed under microscope for observing the presence of various endophytes. A minimum of 100 sections were observed for each sample. Gram's staining was

performed for recording observations on bacterial endophytes. Endophytes were also observed in root segments that were stained for assessing mycorrhizal colonization. Per cent colonization by endophytes was calculated as described for AM colonization.

Data analysis

Data were analyzed following standard methods. Mean values were analyzed by one way ANOVA using Microsoft excel (Microsoft Corp.) for significant differences at $p \leq 0.05$.

Results

Chemical analysis of soil

Results on chemical analysis and pH of soil are presented in Table 2. Values for phosphorus content ranged between 0.05 to 0.07 and 0.06 to 0.09 %, while for carbon content values ranged between 2.39–3.24% and 1.34–2.09% in rhizosphere soil of Group A and Group B trees, respectively. The pH ranged from 6.39 to 7.56 and 7.16 to 8.00, in rhizosphere soil of Group A and Group B trees, respectively.

Table 2 Soil chemical parameters of study sites

Soil characters	Group A (Young trees)			Group B (Old trees)		
	Nainital	Nainital	Kalika	Nainital	Chaubatia	Kalika
Phosphorus (%)	0.07 $\pm$ 0.01	0.07 $\pm$ 0.02	0.05 $\pm$ 0.01	0.06 $\pm$ 0.01	0.07 $\pm$ 0.02	0.09 $\pm$ 0.01
Carbon (%)	2.39 $\pm$ 0.06	3.24 $\pm$ 1.1	2.47 $\pm$ 0.05	1.34 $\pm$ 0.05	2.09 $\pm$ 0.08	1.18 $\pm$ 0.15
pH	7.55 $\pm$ 0.06	7.56 $\pm$ 0.13	6.39 $\pm$ 0.09	8.00 $\pm$ 0.06	7.26 $\pm$ 0.03	7.16 $\pm$ 0.09

Value represents mean $\pm$ SE, $n = 3$

Table 3 Comparison between the microbial counts in the rhizosphere of *G. biloba* of two age groups with that of their corresponding non-rhizosphere soil and the R:S (Rhizosphere: Soil) ratios

Microorganisms (CFU $\times 10^6$)		Group A (Young trees)			Group B (Old trees)		
		Nainital	Nainital	Kalika	Nainital	Chaubatia	Kalika
Bacteria	R	38.67 $\pm$ 0.62	33.00 $\pm$ 0.82	13.00 $\pm$ 0.47	26.30 $\pm$ 0.48	41.33 $\pm$ 1.29	49.33 $\pm$ 0.75
	S	26.33 $\pm$ 0.71	22.67 $\pm$ 0.79	11.00 $\pm$ 0.71	23.30 $\pm$ 1.18	22.33 $\pm$ 0.48	24.67 $\pm$ 1.27
	R:S	1.47	1.45	1.18	1.13	1.85	1.99
Fungi	R	14.67 $\pm$ 1.05	15.30 $\pm$ 0.93	7.67 $\pm$ 0.67	47.3 $\pm$ 1.10	24.00 $\pm$ 0.82	42.00 $\pm$ 0.90
	S	10.00 $\pm$ 0.79	7.33 $\pm$ 0.48	6.33 $\pm$ 0.53	31.0 $\pm$ 1.20	16.33 $\pm$ 1.06	23.33 $\pm$ 1.44
	R:S	1.47	2.09	1.21	1.52	1.47	1.80
Actinomycetes	R	0.67 $\pm$ 0.25	1.00 $\pm$ 0.33	0.33 $\pm$ 0.25	1.33 $\pm$ 0.25	1.33 $\pm$ 0.41	2.33 $\pm$ 0.05
	S	0.33 $\pm$ 0.25	0.67 $\pm$ 0.36	0.33 $\pm$ 0.25	1.00 $\pm$ 0.00	0.67 $\pm$ 0.25	0.67 $\pm$ 0.25
	R:S	2.03	1.49	1.00	1.33	1.98	3.48

Value represents mean $\pm$ SE, $n = 3$

Table 4 Spore density of AM fungi and per cent colonization by AM and other endophytic fungi in the rhizosphere of *G. biloba* of two age groups

Parameters	Group A (Young trees)			Group B (Old trees)		
	Nainital	Nainital	Kalika	Nainital	Chaubatia	Kalika
% AM colonization	39.30 ± 0.62	40.70 ± 0.72	35.70 ± 0.53	18.00 ± 0.77	15.70 ± 0.53	21.30 ± 0.71
Spore density of AM fungi	96.67 ± 0.76	98.67 ± 1.30	34.67 ± 0.78	24.33 ± 0.41	21.00 ± 0.47	27.00 ± 0.67
% colonization by endophytic fungi	78.33 ± 1.04	89.00 ± 1.11	72.00 ± 1.23	51.66 ± 1.17	47.33 ± 1.10	59.66 ± 1.22

Value represents mean ± SE, n=3.

General microflora

Microbial populations recorded for three groups of microbes viz. bacteria, actinomycetes and fungi in both the Groups of trees are presented in Table 3. In general, microbial populations for all the three groups of microbes were higher (statistically significant), in the rhizosphere of Group B trees. Amongst three groups of microbes, bacteria were the most dominant group in the rhizosphere, followed by fungi and then actinomycetes. Highest bacterial (49.33 ± 0.75) and actinomycetes counts (2.33 ± 0.05) were recorded in the rhizosphere of *G. biloba* (Group B) growing at Kalika, whereas, highest fungal counts were recorded in the rhizosphere of *G. biloba* (47.3 ± 1.10) growing at Nainital (Group B).

G. biloba trees of both groups in general exhibited higher microbial populations in comparison of their corresponding non-rhizosphere populations. Among two groups, microbial populations were higher in case of Group B trees. The rhizosphere to soil ratios in Group A trees ranged between 1.18 to 1.47 for bacteria, 1.21 to 2.09 for fungi and 1.00 to 2.03 for actinomycetes. Higher R:S ratios were recorded in most cases in Group B trees, ranging from 1.13 to 1.99 for bacteria, 1.47 to 1.80 for fungi and 1.33 to 3.48 for actinomycetes.

AM colonization

AM fungal colonization and spore density recorded for both the Groups are presented in Table 4. Younger plants of *G. biloba* (Group A) showed higher AM colonization over their older counter parts (Group B). Maximum AM colonization was recorded in younger *G. biloba* (Group A tree) of Nainital (40.70 ± 0.72) and the lowest colonization among the older trees (Group B) was recorded in *G. biloba* of Chaubatia (15.70 ± 0.53). Difference in colonization of AM fungi between the two groups of trees was statistically significant at $P \leq 0.05$ as analyzed by one-way ANOVA. AM colonization was observed mostly in the form of loose intracellular coils while intercellular hypha was rarely found. Absence of arbuscules and presence of few numbers of vesicles were observed.

Data recorded on spore density also showed the same pattern with maximum number of spores (98.67 ± 1.30) in the rhizosphere of *G. biloba* (Group A trees) of Nainital. Significantly higher spore density was recorded in Group A trees as compared to the Group B trees. In most cases, the spores were devoid of hyphal attachment. Based on the morphological characters, spores isolated from the rhizosphere soil were identified upto genus level. All the spores belonged to the genus *Glomus* with five different types and tentatively designated as *Glomus* sp. A, B, C, D and E. *Glomus* sp A, B, C, and D are shown in Fig.1.

Endophytic organisms

Presence of endophytes was observed in many cases throughout this study (Fig. 2). Colonization of endophytic fungi was found to be 2–3 times higher as compared to AM fungi (Table 4). The most striking and interesting observation was presence of bacterial endophytes in cortical cells of primary as well as secondary roots of *G. biloba* of both the Groups (Fig. 2D). The observed bacteria were gram-negative oval rods as revealed by gram's staining. The presence of endophytic bacteria was observed in transverse sections of the roots. Endophytes observed could not be identified in the course of this study due to the problems in culturing of these organisms. This work is still under progress.

Data recorded on two age groups of *G. biloba* revealed an inverse correlation between per cent AM colonization and colonization of general microflora. When the populations of general microflora (bacteria, fungi and actinomycetes) were higher as in case of Group B trees, the AM colonization was poorer and vice versa.

Discussion

In the present study, estimations of general microbial populations, colonization of AM fungi, and observations on endophytic organisms associated with *G. biloba* of two age groups have been carried out. The rhizosphere of *G. biloba* in general was stimulatory for microbial populations. The

Fig. 1 AM spores belonging to genus *Glomus* (A–D) detected from the rhizosphere of *G. biloba* and colonization of roots by AM fungi; extraradicle (E) and intraradicle hyphae (F). Bar = 40 μ m.

only exception was in case of actinomycetes where the R:S ratios were below one in case of Group A trees. Every plant species provides an individual and specific site of microbial activity in form of a rhizosphere. Besides various biotic and abiotic factors and plant age seem to play an important role in determination of rhizosphere microflora. In general, the rhizosphere effect is expressed by greater microbial activity, and bacteria are the group most stimulated by the rhizosphere. In the present study also, similar observa-

tions were made; bacteria were the dominant group in the *G. biloba* rhizosphere, significantly higher as compared to non-rhizosphere microbial counts. The classical concept of rhizosphere effect which describes the stimulation of microbial populations has been based on the results of research conducted largely on short duration plants and only a few tree species [9, 10]. While in some of the recent studies, that were conducted on rhizosphere of tea plantations and several tree species of the IHR region, the

Fig. 2 Fungal endophytes in root segments (A, B, C) and bacterial endophyte in transverse section (D) of the root of *G. biloba*. A and B, C, D represents X 200 and X 400 magnifications, respectively.

rhizosphere effect of both stimulatory as well as suppressive nature has been reported [11, 12]. In case of tea where the negative rhizosphere effect was reported from older tea bushes, besides several other factors, lowering in pH of soil with increasing age was considered as one of the important factors affecting the soil microbial populations [13]. In *G. biloba* rhizosphere, where the soil pH ranged between 6.39–8.00, could be one of the factors for maintaining the modest microbial populations. Higher bacterial populations, recorded in older trees (except at Nainital site) could be accounted to the influence of higher P% level in rhizosphere soil. At Nainital, lowering in P content in soil in comparison to the other two sites could have accounted for lesser bacterial counts. Besides, slightly alkaline conditions (8.0) at this site could also have suppressed the bacterial population. Bacteria are known to grow optimally between pH 6.5–7.5.

In the present study the age of the *G. biloba* appeared to affect the AM fungal colonization. The Group B trees showed poor root colonization and lower spore density as

compared to Group A trees. The low level of colonization found restricted to short absorbing rootlets invaded at their base and the structural similarities were found as in an earlier study carried out on colonization of *G. biloba* roots by vesicular-arbuscular mycorrhizae by Fontana [14] in natural and controlled conditions. The composition of AM fungal community may be strongly affected by individual plant species through differential effects on hyphal growth and sporulation [15, 16]. It was interesting to note that while species of only one genus i.e., *Glomus* constituted the spore population in *G. biloba* rhizosphere, other tree species including *Taxus baccata* and five species of rhododendrons growing in the similar ecological niches of IHR have recently been reported to harbour many other genera of AM fungi [17, 18].

Although numerous fungi are known to form mycorrhizae in a particular host tree, the species composition of the mycorrhizal flora is not constant and a temporal shift in kinds and in number of fungi may occur as tree age [19]. The density of AM fungi as measured by per cent

colonization and total spore count, first increased with time since abandonment and then decreased in the late successional forest sites [20]. Our results on two age groups of *G. biloba* trees can be considered as early and late successional stages, respectively, show consistency with the above.

Generally, AM fungi are known to favour the growth of beneficial microbes and both AM fungi and other microbes are known to influence each other [21, 22]. In the present study, contrasting results were obtained for AM fungi and other microbial population. While AM fungal population was higher in Group A (young) trees, other microflora was higher in Group B (older) *G. biloba* trees. These results are difficult to compare since such studies including both AM fungi and normal microflora are deficient. This observation can be considered specific for *G. biloba* rhizosphere where AM fungi can be helpful in plant establishment during early phases and other microbial flora may provide consistent benefits during prolonged time. Negative correlations of microbial counts with per cent colonization of AM fungi were obtained and not with spore density in Group A trees of *G. biloba*. It can be considered that these rhizosphere microflora somehow inhibit the germination of AM spore thereby resulting in lower colonization by AM fungi. Rhizosphere microorganisms have been known to affect the pre-symbiotic stages of the AM fungal development, such as spore germination and germ tube development [23, 24]. After prolonged period, in old trees, spore density decreased significantly as compared to younger plants due to a continuous restriction on the AM colonization which further, lead to poorer colonization in old *G. biloba* trees. This is supported by the negative correlation between per cent AM fungal colonization and bacterial and actinomycetes counts. Also, negative correlation was recorded between AM fungal spore density and bacterial counts.

Observations on occurrence of endophytic bacteria and fungi are important features of this study. Algal [25] and fungal [26] endosymbionts have been previously reported from *G. biloba* roots but bacterial endosymbiont is being reported for the first time to the best of our knowledge. Occurrence of endophytes that could have played important linkages in evolutionary biology is getting attention also due to their recognition for some biotechnological applications [27]. A fungus *Xylaria* sp having broad antimicrobial activity against both bacteria and fungi has been isolated from *G. biloba* [26]. Screening of isolated microbes for the plant growth promoting traits may be useful. The present study is in progress; the bacterial endosymbiont has been isolated and based on the morphological, biochemical and physiological characters tentatively identified as *Pseudomonas* sp. Characterization of this isolate with special reference to its plant growth promoting properties is in progress. Use

of such microorganisms can be beneficial for the propagation of this medicinally important plant by tissue culture or conventional cutting methods where hardening of plants by biological means can improve plant survival and further growth. Other biotechnologically important traits like production of some pharmaceutically important molecules may also be explored for the potential uses.

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