Front. Biol. 2014, 9(1): 82–88
DOI 10.1007/s11515-014-1292-4

RESEARCH ARTICLE

Influence of ectomycorrhizal inoculation on *Pinus wallichiana* and *Cedrus deodara* seedlings under nursery conditions

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Abstract **A study was undertaken to examine the extent of root colonization by four locally isolated ectomycorrhizal (ECM) fungi (*Hebeloma theobrominum, Boletus dryophilus, Scleroderma citrinum* and *Suillus luteus*) and their effects on seedling growth in *Pinus wallichiana* and *Cedrus deodara* under nursery conditions. Seedlings of the two conifers were inoculated with mycelium of ECM fungi and were grown in pots containing sterilized forest soil for six months. The percentage of ECM colonization of roots was 38%–52% in *Pinus wallichiana* and 33%–48% in *Cedrus deodara*. ECM colonization increased shoot height, needle number, shoot and root biomass and survival of inoculated seedlings. Among the four ECM fungi *Hebeloma theobrominum* was more effective with *Pinus wallichiana* and *Scleroderma citrinum* with *Cedrus deodara* in promoting seedling survival and overall growth. All the four ECM fungi used enhanced growth of inoculated seedlings and thus can be used in afforestation and regeneration programmes in degraded forests ecosystems.**

Keywords afforestation, colonization, ectomycorrhizal, mycelium, seedling survival

Introduction

Ectomycorrhizas are mutualistic symbiotic associations formed between fine roots of forest trees with certain soil fungi mostly basidiomycetes and some ascomycetes. ECM fungi are particularly essential to the health and growth of forest trees and benefit their associated forest trees in a number of ways, the most important being enhancement of soil nutrient uptake, particularly for elements with a low mobility in the soil, such as phosphorus (P), nitrogen (N) and several micronutrients (Smith and Read 2008; Kemppainen and Pardo, 2010). Keeping their beneficial effects in view, species of ECM fungi have been used in a number of studies designed to evaluate the survival and growth of out planted ECM tree seedlings. The response of tender out planted seedlings to mycorrhizal inoculation is positive, neutral, or negative depending on environmental factors or on ECM species or host plant used (Sharma et al., 2008). Fungal colonization of root systems is an important factor in determining seedling vigour and, consequently, their quality (Smith and Read, 2008). Several mycorrhizal fungi have been shown to have a positive impact on seedling health and productivity in forest nurseries (Steinfeld et al., 2003; Parladé et al., 2004). Furthermore, following the transfer and out planting of the seedlings into the field, mycorrhizal fungi promote survival, establishment and growth of young trees in newly established forest plantations (Turjaman et al., 2006). The main mechanisms for this are thought to be enhanced uptake of nutrients and water, lengthened root life, buffer against various stresses, and increase resistance against some pathogens (Bois et al., 2005; Turjaman et al., 2006; Quoreshi et al., 2008; Smith and Read, 2008).

The lack of mycorrhizal associations on plant root systems is one of the major reasons for failure of plantation establishment and growth in various forests with low inoculum potential, mined sites, and highly disturbed areas. This is because ectomycorrhizal inoculum can persist for a short time after disturbance as chlamydospores, sclerotia, on root tips of surviving trees, and, briefly, as hyphae emanating from dying or recently dead root tips. Ectomycorrhizal fungi generally cannot survive in the soil for long periods as the hyphae are typically attached to living roots. Thus, without a host, the amount and diversity of ECM fungal inoculum in the soil decreases rapidly (Dahlberg, 2002; Baar et al., 2002;

Received September 19, 2013; accepted December 14, 2013
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Jones et al., 2003), with greater declines following more severe disturbances (Bradbury et al., 1998). The recovery of ectomycorrhizal fungi following a disturbance takes time, usually decades (Visser 1995). Consequently, failure in afforestation has been attributed to the absence of suitable mycorrhizal fungi in the soil (Nuñez et al., 2009). One way of overcoming this problem is to pre-inoculate seedlings with suitable mycorrhizal fungi, and this possible option has been investigated in several recent studies (Dunabeitia et al., 2004; Alguacil et al., 2005, Caravaca et al., 2005; Teste et al., 2009). Many recent studies have also shown that seedling survival (McGuire, 2007), growth (Nara, 2006; Turjaman et al., 2006; Teste and Simard, 2008; Dar et al., 2010), ECM fungus colonization (Nara and Hogetsu, 2004; Turjaman et al., 2006), and ECM fungus diversity (Cline et al., 2005; Teste et al., 2009) are greater where seedlings establish within the mycorrhizal network of mature trees. Thus, it becomes evident that role of ectomycorrhizal fungi is critical to the survival of seedlings planted for restoration and rehabilitation of degraded forest ecosystems.

The Jammu and Kashmir state located in the far north of India is a mountainous area in the north-west Himalayas that shares international boundaries with Pakistan in the west and China in the north-east. The state has only 14% of its area under forest cover; of which conifers occupy 41% area and rest are covered by broad leaves forest plants. Conifers, especially *Pinus wallichiana* and *Cedrus deodara*, are economically very important trees. *Cedrus deodara* is strongest of the Indian coniferous woods and is one of the most valuable Indian timbers. *Pinus wallichiana* and *Cedrus deodara* show complete mycorrhizal dependence in early sapling establishment and survival. The present study was aimed to evaluate the influence of some locally isolated indigenous ectomycorrhizae on *Pinus wallichiana* and *Cedrus deodara* seedlings under nursery conditions.

Materials and methods

Seed and plant substrates

To study the effect of ectomycorrhizal inoculation on growth and survival of *Pinus wallichiana* and *Cedrus deodara* seedlings nursery experiment was set up at Kashmir University Botanic Garden (KUBG) and Mammar Forest Department Nursery (J&K). The seeds of the two conifers were collected from the mature trees of *Pinus wallichiana* and *Cedrus deodara* in the forest. The soil used for the pot experiment was collected from mature mixed forest in Kangan, Kashmir, India, and was sterilized in an autoclave for one hour. A preliminary experiment showed that this sterilization procedure got rid of most of the ECM and pathogenic fungi. Seeds of the two conifers were soaked for 12 h in sucrose added tap water and gently washed with running water. These seeds were sown in polyethylene pots (bags) (size 10 cm × 10 cm) containing 500 g sterilized soils. Pots containing seeds were transferred to a nursery at Kashmir University Botanic Garden (KUBG) and Mamar Forest Department Nursery (J&K). One seedling was grown per pot under a 75% shading intensity net to control solar radiation.

Ectomycorrhizal inoculum

Mycelial inoculum of four ECM species (*Hebeloma theobrominum, Boletus dryophilus, Scleroderma citrinum* and *Suillus luteus*) was used to study the effect of ectomycorrhizal inoculation on *Pinus wallichiana* and *Cedrus deodara* seedlings under nursery conditions. The mycelial inoculum was obtained from the sporocarps of these four ECM species and for this purpose ectomycorrhizal sporocarps were cultured on synthetic medium. Isolation of pure cultures was done under sterile conditions in a laminar flow hood. Only fresh, un-infected sporocarps were used for isolation of cultures. Small amounts of fungal tissue were removed with flame sterilized razor blades and forceps and placed on agar medium in a Petri dish. Tissue was removed from the apex of the stem or the cap itself (Brundrett et al., 1996). Media used for culturing was modified Melin Norkans media (MMN). Streptomycin was used as bacteriocide and added to the medium in concentrations of 50 mg per liter (1000 mL) of medium. The medium was autoclaved at a pressure of 25 psi and temperature of 121°C. The pH of the medium was set at 5.5–5.8. Cultures were incubated at 25°C in the dark and checked daily for contamination and growth during the initial 2 weeks of incubation. Purity of isolated cultures was confirmed by comparing RFLP patterns of amplified ITS regions of culture with that of fruit body. Two restriction enzymes, namely Alu1 and BamH1 were used for RFLP comparison.

The isolated cultures were sub cultured on broth MMN medium. From these plates, agar plugs (approximately 0.5 cm diameter) were removed from the edges of all the colonies and placed in one 250 mL screw-capped Erlenmeyer flask containing 50 mL of MMN medium with glucose concentration 10 g/L. The loosely capped flasks were incubated for 21 days at 25°C. The recovered biomass was washed with 3 volumes of distilled water and then dried at 80°C until constant weight was achieved.

Mass inoculum production

The mass inoculum for bioreactor was prepared in MMN broth. The mycelial inoculum was homogenized for 10 s under sterile conditions. Homogenate formed was used to inoculate Biostat®B bioreactor containing 3L MMN medium (Glucose 10 g/L) at the rate of 200 mL of inoculum to 3L of Growth Medium (6% v/v inoculum) under aseptic conditions. Batch fermentation (Fig. 1) was carried out at temperature 23 ±2°C, pH 5.8 and 100 rpm speed. The air flow rate was 1.0–

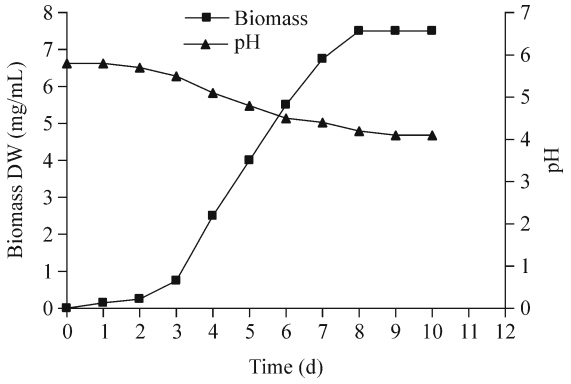

Figure 1 *Hebeloma theobrominum* growing in bioreactor after 8 days of incubation.

2.0 L/min, which was supplied through a 0.2-µm Millipore filter. The maximum biomass (dry weight) achieved in the airlift bioreactor was approximately 7.5 mg/mL after 8–10 days of incubation (Fig. 2). The period of fermentation was 11 days, until the glucose in the medium was completely exhausted.

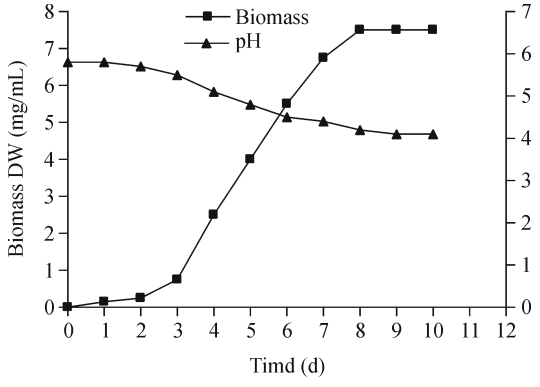

Figure 2 Biomass profile of *Hebeloma theobrominum* for batch fermentation using MMN medium. Conditions: Glucose 10 g/L; Temperature 23.0±2°C; Initial pH 5.8; Shaking speed 100 rpm; Air flow rate 1.5±0.5 L/min; inoculum size 6% (v/v).

Ectomycorrhizal inoculation

Inoculation of seedlings was carried out 20 days after germination of seeds, and for inoculation 10 mL of mycelial inoculum was applied to each germinated seedling (i.e. 75 mg of mycelial inoculum). Inoculation was performed by removing the top soil (3–5 cm) from the bags and sprinkling the mycelium around the roots using micropipette. Fresh autoclaved forest soil was again layered over the inoculum. The seedlings were irrigated with tap water every day or more depending on weather conditions and weeds were removed continuously from seedlings. There were fourty seedlings of each fungus-host plant combination. The study field trial was monitored regularly after every 20 days and seedling survival, shoot height and number of needles per seedling was recorded. The following treatments were used: (1) control, (2) *Hebeloma theobrominum* inoculated, (3) *Boletus dryophilus* inoculated, (4) *Scleroderma citrinum* inoculated, and (5) *Suillus luteus* inoculated.

Shoot and root biomass, and mycorrhizal colonization were studied after six months of inoculation of seedlings. To calculate the percentage of ECM colonization, roots were cleaned using running water to separate them from the soil and then the root systems were spread on trays. The total number of root tips and the number of ECM short roots were counted under a dissecting microscope. Verification of ECM colonization was obtained by examining the cross section of root tips (cut manually) under a compound microscope for the presence of mantle and Hartig net (Brundrett et al., 1996). Biomass of shoot and root was assessed after the plants were oven-dried at 110°C for 24 h. The average Shoot height, Needle number and Shoot and Root biomass was calculated from the value of fourty replicate seedlings for each fungus treatment and analyzed statistically using ANOVA with $P = 0.05$.

Results

Ectomycorrhizal colonization

After the end of the six months in the nursery, all ectomycorrhizal fungi tested formed ectomycorrhizas on *Pinus wallichiana* and *Cedrus deodara* seedlings (Table 1). All ectomycorrhizas associated with roots of *Pinus wallichiana* and *Cedrus deodara* were of the inoculants type, and there was no cross-contamination between treatments. ECM colonization of *Pinus wallichiana* and *Cedrus deodara* seedlings inoculated with mycelium of ECM fungi was not significantly different among the four different fungi but significant difference was observed in colonization between the two conifers. Among the two conifers, colonization percentage was higher in *Pinus wallichiana* and lower in *Cedrus deodara*. Among the four ECM fungi, *Hebeloma theobrominum* was more effective with *Pinus wallichiana* and *Scleroderma citrinum* with *Cedrus deodara*. Control seedlings (uninoculated) were also colonized by indigenous ECM fungi to some extent but colonization percentage was much lower than inoculated seedlings.

Plant growth and survival

Ectomycorrhizal colonization of *Pinus wallichiana* and *Cedrus deodara* seedlings increased plant growth and survival of seedlings. All the four ECM used in the present study increased the height of shoot, needle number of shoots, biomass of shoots and roots and seedling survival of *Pinus wallichiana* and *Cedrus deodara* (Tables 1and 2). The data was analyzed statistically by ANOVA and the difference was found to be significant at $P = 0.05$ as F value obtained was found to be higher than F critical value. Furthermore, there

Table 1 Effect of ECM inoculation on ECM colonization, seedling survival, height, biomass and needle no. in *Pinus wallichiana* under nursery conditions

Treatment	ECM colonization (%)	Seedling survival (%)				Seedling height (cm)				Biomass (mg)		Needle no.			
	180d*	20d	40d	60d	180d	20d	40d	60d	180d	Shoot	Root	0d	20d	40d	60d
Control	18	72	62.5	50	35	3.2±0.139	5.76±0.156	6.88±0.121	10.45±0.387	170.33±1.059	29.55±1.009	10.27±0.266	11.15±0.345	12.92±0.334	14.25±0.455
Hebeloma theobrominum	52	92.5	90	77.5	68	4.2±0.660	8.56±0.140	9.55±0.186	13.86±0.149	270.45±1.408	48.45±1.993	11.92±0.266	14.82±0.332	16.5±0.328	19.95±0.665
Boletus dryophilus	42	92	85	75	62	5.1±0.136	7.3±0.177	8.45±0.109	12.86±0.282	246.54±2.461	40.45±1.797	10.33±0.392	17.05±0.363	18.29±0.380	24.73±0.611
Scleroderma citrinum	48	57	75	70	57	4.0±0.138	7.56±0.106	8.2±0.175	13.42±0.228	258.86±2.874	45.33±1.400	12.24±0.378	16.5±0.386	18.3±0.531	26.00±0.633
Suillus luteus	38	54	75	65	54	3.9±0.168	6.75±0.184	7.9±0.133	12.48±0.309	230.32±1.376	37.45±1.379	9.35±0.323	17.35±0.399	19.2±0.435	25.7±0.529
P = 0.05	–	–	–	–	–		*F* = 5.267 / F critical 3.259			*F* = 2.776	*F* = 2.776		*F* = 5.466/ F critical 3.259		

d* = days after inoculation (Mean±S.Em is based on fourty replicates for each fungus treatment).

Table 2 Effect of ECM inoculation on ECM colonization, seedling survival, height, biomass and needle no. in *Cedrus deodara* under nursery conditions

Treatment	ECM colonization (%)	Seedling survival (%)				Seedling height (cm)				Biomass (mg)		Needle no.			
	180d*	20d	40d	60d	180d	20d	40d	60d	180d	Shoot	Root	0d	20d	40d	60d
Control	14	85	72.5	65	48	2.9±0.072	4.2±0.106	5.48±0.085	7.46±0.083	147.33±0.927	20.55±0.898	17.47±0.498	18.65±0.402	19.33±0.516	20.24±0.627
Hebeloma theobrominum	33	92.5	87.5	87	76	3.3±0.041	5.6±0.055	7.55±0.077	9.55±0.109	173.32±1.464	24.45±1.566	17.47±0.359	22.82±0.460	25.82±0.590	28.32±0.908
Boletus dryophilus	40	85	85	75	68	3.5±0.094	4.8±0.174	6.45±0.126	10.83±0.334	188.54±1.714	25.45±1.347	18.3±0.409	20.9±0.638	27.23±0.765	33.34±0.737
Scleroderma citrinum	48	90	85	75	65	2.9±0.1.4	4.5±0.052	6.2±0.089	11.86±0.069	228.86±1.859	32.45±2.550	17.4±0.343	25.2±0.490	28.5±0.594	36.33±0.760
Suillus luteus	38	80	75	70	60	3.2±0.066	4.8±0.143	5.9±0.069	9.83±0.166	222.45±3.121	30.32±1.859	16.2±0.528	24.5±0.759	27.9±0.893	35.32±0.952
P = 0.05	–	–	–	–	–		*F* = 3.490 / F critical 3.259			*F* = 2.776	*F* = 2.927		*F* = 4.397 / F critical 3.259		

d* = days after inoculation (Mean±S.Em is based on fourty replicates for each fungus treatment).

was no significant difference in plant growth between fungal treatments but significant difference was observed between control and fungus inoculated seedlings. *Hebeloma theobrominum* was much more effective than other three ECM fungi in enhancing seedling survival of both the conifers. Needle number per seedling was increased by *Suillus luteus* in *Pinus wallichiana* and by *Scleroderma citrinum* in *Cedrus deodara*. Shoot height and biomass was found to be increased by *Hebeloma theobrominum* inoculation in *Pinus wallichiana* and by *Scleroderma citrinum* in *Cedrus deodara*.

Discussion

All the four ECM species tested formed mycorrhizal association with roots of *Pinus wallichiana* and *Cedrus deodara* seedlings at the end of the six-month study period under nursery conditions. ECM colonization increased plant growth and survival of seedlings and significant difference in various parameters was observed between fungus inoculated and control seedlings. Survival percentage of seedlings was highest among all the four fungus inoculated seedlings as compared to control seedlings. Among the four ectomycorrhizal fungi *Hebeloma theobrominum* was more effective and *Suillus luteus* was least effective in promoting seedling survival of the two conifers. With *Pinus wallichiana* the present study of mycorrhizal root colonization and their influence on plant growth revealed an increase of 19%–32%, 35%–58%, 26%–63% in plant height and shoot and root biomass, respectively, in comparison to uninoculated control seedlings. Maximum plant height and higher root colonization was observed in *Hebeloma theobrominum* inoculated plants followed by *Scleroderma citrinum* and *Boletus dryophilus* (Table 1). The plants inoculated with mycorrhizae showed more increase in height and biomass with age as compared to control. The pine plants attained maximum height of 13.86 cm after 180 days of inoculation in case of *Hebeloma theobrominum* which was 32% higher than control. This was followed by 13.42 and 12.86 cm height observed in plants inoculated with *Scleroderma citrinum* and *Boletus dryophilus,* respectively. Highest shoot and root dry biomass of 270.45 and 48.45 mg·plant^{-1}, respectively, was observed in plants inoculated with *Hebeloma theobrominum* which was 58% and 63% higher than control. This was followed by 258.86 and 45.33 mg·plant^{-1} in *Scleroderma citrinum* and 246.54 and 40.45 mg·plant^{-1} in *Boletus dryophilus*. Needle number per seedling 26.00 was maximum in *Scleroderma citrinum* followed by 25.7 in *Suillus luteus* and 24.73 in *Boletus dryophilus* inoculated seedlings. Highest root colonization of 52% was recorded in *Hebeloma theobrominum* followed by 48% in *Scleroderma citrinum* and 42% in *Boletus dryophilus.*

In case of *Cedrus deodara* seedlings, mycorrhizal inoculation resulted in 28%–58%, 17%–55% and 18%–57% increase in plant height and shoot and root biomass, respectively, in comparison to uninoculated control. Maximum increase was noticed in *Scleroderma citrinum*inoculated plants followed by *Boletus dryophilus* and *Suillus luteus* inoculated plants which attained a height of 11.86, 10.83 and 9.83 cm, respectively, after 180 days of inoculation in comparison to the lowest of 7.46 cm in control (Table 2). Maximum shoot and root dry biomass of 228.86 and 32.45 mg·plant^{-1} was recorded in *Scleroderma citrinum* inoculated deodar plants, followed by *Suillus luteus* (222.45 and 30.32 mg·plant^{-1}) and *Boletus dryophilus* (188.54 and 25.45mg ·plant^{-1}). Needle number per seedling 36.33 was maximum in *Scleroderma citrinum* followed by 35.32 in *Suillus luteus* and 33.34 in *Boletus dryophilus* inoculated seedlings. Highest root colonization of 48% was recorded in *Scleroderma citrinum* followed by 40% in *Boletus dryophilus* and 38% in *Suillus luteus.* The findings are in agreement with those of Turjaman et al. (2006) who observed variable mycorrhization in *Shorea seminis* seedlings with the inoculation of *Pisolithus arhizus* and *Scleroderma columnare.* Similarly, Parladé et al. (2004) observed positive correlation between mycorrhizal infection of *Rhizopogon* sp. with seedling growth, mycorrhizal root colonization and survival in *Pinus pinea* and *P. halepensis* seedlings. Wang et al. (1985) reported that ectomycorrhizae increased the biomass production of pine seedlings (*Pinus tabulaeformis*) by about 40% with the inoculation of *Boletus* sp., *Suillus greivilli* and *P. tinctorius.* Similarly, Dar et al. (2010) also reported significant increase in plant biomass, shoot height, root length and mycorrhizal root colonization in deodar and pine seedlings due to inoculation of *Tricholoma album*, *Hygrophorus camrophyllus*, *Suillus granulatus*, *Boletus edulis* and *Scleroderma* sp..

The present study revealed that the percentage of ECM colonization on *Pinus wallichiana* and *Cedrus deodara* was higher by 34% than control. *Scleroderma citrinum*had higher ECM colonization with *Cedrus deodara* and *Hebeloma theobrominum* with *Pinus wallichiana*. Turjaman et al. (2006) also reported increase in ECM colonization in *Shorea seminis* seedlings with the inoculation of *Pisolithus arhizus* and *Scleroderma columnare* under field conditions. Early colonization of conifer seedlings was highly dependent on contact with living ECM roots of adult trees (Alexander et al., 1992). This suggests that controlled inoculation of conifer seedlings in the nursery with selected efficient ECM fungal strains should be introduced in forest regeneration programmes. ECM colonization was also observed under control treatments of *Cedrus deodara* (14%) and *Pinus wallichiana* (18%) seedlings in the nursery. Therefore, it is possible that indigenous ECM fungi persisted in the nursery and slowly reached the seedling roots and formed ECM. However, the significant difference of all parameters studied between controls and inoculated seedlings were due to faster colonization by inoculated fungi so that either ECM fungi could prevent invasion by indigenous ECM fungi. The fungi on the control seedlings developed yellowish brown ectomycorrhizal roots, however the indigenous ECM could not compete with inoculated ECM.

Conclusions

In our experiments colonization of roots of *Pinus wallichiana* and *Cedrus deodara* by ECM inoculum increased survival and early growth of seedlings after six months under nursery condition. A positive relationship was found between colonization extent and shoot growth of conifer seedlings inoculated with ECM fungi. With the present interest in establishing regeneration or plantation of *Pinus wallichiana* and *Cedrus deodara* including other members of Pinaceae, mycorrhization is a key technology to enhance or encourage rapid growth of seedlings on a large scale in commercial nurseries and to enable the seedlings to survive successfully in the field. Furthermore, it is important to consider that Kashmir Himalaya serves an important habitat for conifers which occupy 41% of forest area and rest is covered by broad leaves forest plants. Conifers, especially pine and deodar, have great potential as valuable timber and as a source of fiber material for pulp and paper industry and these conifers show complete mycorrhizal dependence in early sapling establishment and survival. The results of this study also showed that inoculation of ECM fungi can increase early growth of *Pinus wallichiana* and *Cedrus deodara* seedlings grown in major forests of Kashmir Himalaya, India and that this technique will accelerate and assure the achievement of reforestation programmes.

Acknowledgements

The authors are highly grateful to the Department of Biotechnology, Government of India for providing financial grant for the conduct of present research.

Compliance with ethics guidelines

Itoo Z A and Reshi Z A declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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