

MOLECULAR CHARACTERIZATION AND *IN VITRO* EVALUATION OF INDIGENOUS *SUILLUS* ISOLATES FOR THE PRODUCTION OF MYCORRHIZAL BLUE PINE (*PINUS WALLICHIANA*) SEEDLINGS

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ABSTRACT

Fresh basidiocarps of *Suillus* species were collected from conifer forests of the northwestern Himalayan region of India during monsoon seasons. Eight pure cultures were obtained from the basidiocarps of a range of *Suillus* species. Internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) genes of all the *Suillus* isolates obtained were amplified. Variations within the amplified ITS region of the rDNA genes of *Suillus* isolates were examined by restriction fragment length polymorphism (RFLP). Inter-specific variations in the length and number of restriction sites within the ITS region were observed. Restriction enzyme digests of the ITS–rDNA products for eight *Suillus* isolates separated the isolates into five different groups. When compared the ITS sequences with existing database and the RFLP patterns, the *Suillus* species were reliably distinguished into five different species, namely *S. sibiricus*, *S. granulatus*, *S. triacicularis*, *S. himalayensis* and *S. indicus*. In addition, some physiological attributes of all the *Suillus* isolates, such as radial growth, biomass yield and *in vitro* mycorrhizal capacities were evaluated to select efficient native fungal inocula for the production of mycorrhizal blue pine (*Pinus wallichiana*) seedlings in nursery. Inter-specific and intra-specific variations were observed in radial growth, biomass yield and mycorrhizal capacities of different *Suillus* isolates. Furthermore, the effects of fungal isolates on growth and biomass yield of *P. wallichiana* seedlings were assessed after four months of the mycorrhizal inoculation. All the *Suillus* isolates enhanced the growth and biomass yield of *P. wallichiana* seedlings as compared to the control treatment, but at different rates. *Suillus sibiricus* isolate SNW06 showed highest improvement in plant growth, biomass and concentration of most nutrients, whereas *S. himalayensis* isolate SNW03 was found to be least effective. On the basis of physiological analysis, mycorrhizal colonization and growth response of *P. wallichiana* seedlings, *S. sibiricus* isolate SNW06 was found to be the most effective *Suillus* isolate for mycorrhizal inoculation of *P. wallichiana* seedlings in nurseries and experimental plantations, followed by *S. indicus* isolate SNW02. Thus, the present study evaluated different indigenous *Suillus* isolates that are best adapted to the local environmental conditions and led to the selection of native and efficient ectomycorrhizal strains for blue pine afforestation programmes.

Keywords: ectomycorrhizal fungi, Himalayan, ITS, *Pinus wallichiana*, *Suillus*

INTRODUCTION

Ectomycorrhizal (ECM) fungi generally improve growth and survival of host plants, increase their nutrient and water uptake, and provide them resistance against biotic (e.g. plant pathogens) and abiotic (e.g. heavy metals) stresses [1-3]. There are many studies demonstrating the positive effect of ECM fungi on growth and nutrient contents of plants, especially the pine trees [4-11]. While considering the ECM *Suillus* species, the fungi have been shown to promote plant height, root shoot biomass as well as nitrogen and phosphorus uptake in pine trees [12-16]. Furthermore, *Suillus* isolates exhibit metal tolerance to many toxic metals, such as Zn, Cu and Cd [17-19] and these metals tolerant *Suillus* isolates have been shown to protect mycorrhizal pine seedlings from metal stress [19-22]. Thus, *Suillus* isolates can be used as an excellent mean for large-scale afforestation and regeneration of pine seedlings.

The ECM fungi differs in their physiological attributes, such as morphology, growth rates and mycorrhizal ability and this is certainly true for *Suillus* species, which exhibit remarkable inter-specific as well as intra-specific variations for a wide range of physiological traits [14, 23, 24]. On the basis of these differentiating features, suitable and efficient ECM isolates can be selected for mass inoculum production for forestry purposes. *In vitro* mycorrhizal capacity of the different local *Suillus* isolates has been evaluated for the growth of a typical Mediterranean pine species (*Pinus halepensis*) with an aim to select

suitable isolates for afforestation programmes [14]. Although there are few studies reporting positive effects of different ECM fungi on growth, biomass, and nutrient contents of *Pinus wallichiana* seedlings [7, 8, 10, 25], but the studies focusing on isolation and evaluation of mycorrhizal capacity of indigenous *Suillus* species to promote growth of *P. wallichiana* seedlings are still lacking. Owing to different advantages provided by *Suillus* species to the host plant, particularly pine species, the present study aimed at isolation of local *Suillus* species and evaluation of their mycorrhizal capacities to select suitable isolates for mass inoculums production was undertaken.

MATERIALS AND METHODS

Isolation of pure cultures

Modified Melin-Norkrans (MMN) agar media [26], Malt Extract (ME) agar (2% w/v) media and Potato dextrose agar (PDA) media supplemented with streptomycin (50µg/ml) were used to isolate pure cultures. Fresh basidiocarps collected were surface sterilized with rectified spirit and cut along the pileal surface with sterile surgical blades to expose the inner pileal flesh. Two–three pieces of clean fresh pileal flesh were transferred to each agar plate and incubated at 25°C for one month. Plates were checked weekly for any contamination and sub-culturing was done, if required. The pure cultures isolated are being maintained on Malt Extract (ME) agar (2%, w/v) media in our laboratory. All the cultures isolated have been and submitted to Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and the corresponding basidiocarps have been deposited in the Herbarium of the Botany Department (PUN), Punjabi University, Patiala, India.

Molecular studies

For molecular analysis of *Suillus* species, genomic DNA was extracted from isolated cultures according to Zhou *et al.* (1999) [27]. The internal transcribed spacer (ITS) region was amplified by PCR using the universal primers [28]. For sequencing purpose, PCR products were separated by electrophoresis on 1.5% agarose gels and purified using a Gel Extraction Kit. In order to obtain restriction patterns for different *Suillus* isolates, purified ITS products were subjected to RFLP (Restriction fragment length polymorphism) analysis. Three different restriction enzymes, namely *AluI*, *HaeIII* and *MboI* (Fermentas) were used in separate digestion reactions with the amplified ITS products. The digested DNA was electrophoresed through 2.0 % (w/v) agarose gels containing ethidium bromide (0.5 µg/ml) for 4 h at 50 V. The restriction patterns were visualized and photographed using gel documentation system (Quantum–ST4–3026/WL/26M, Vilber Lourmat).

Fungal growth

Growth of *Suillus* cultures isolated was measured on 2% malt extract media with regular sub-culturing. Briefly, fungal plugs (approximately 6 mm diameter) were grown on 90 mm Petri-dishes containing 2% malt extract agar media with three replicates for each isolate. Radial growths of fungal isolates were recorded after incubation at 25 °C in dark for 4 weeks. Biomass yields of above eight isolates in broth media were also studied. On 90 mm malt extract agar plates, a single colony of each fungus was established until they were 2.0–3.0 cm in diameter. From these plates, single agar plug (approximately 6 mm diameter) was removed from the edges of all the colonies and placed in 250 ml screw-capped Erlenmeyer flasks (three replicates for each isolate) containing 25 ml of malt extract broth media. The loosely capped flasks were incubated for 4 weeks at 25 °C in dark conditions. The mycelia were harvested by filtration through pre-weighed filter papers (Whatman No. 1). The mycelia were washed with 3 volumes of distilled water and recovered biomasses were dried at 70 °C until constant weight was achieved.

In vitro mycorrhization

The growth performance of *P. wallichiana* seedlings inoculated with different *Suillus* isolates was evaluated, as described previously by Beatriz *et al.* (2006) with some modifications [14]. In brief, *P. wallichiana* seeds were washed with tap water followed by washing with distilled water and finally surface sterilized in 30% H₂O₂ (v/v) for 25 min in a sterilized flask. The seeds were again rinsed three times with sterilized distilled water, sown in 1.0% water-agar plates and incubated

in slanted position at 25 °C for two weeks. The germination rate was 70–80% after 8-12 days. Pre-germinated seedlings (1–2 cm root length) were transferred into the tubes containing one month grown *Suillus* isolates. For this purpose, tubes (50 cm³) were filled with peat, vermiculite (1:10, v/v) mixture and supplemented with 15 ml of liquid Malt extract (2% w/v) media. Twelve tubes were inoculated with 3–4 mycelia plugs (6 mm diameter) cut from the margin of a three weeks old fungal colony of each *Suillus* isolate and twelve tubes without any fungus were kept as control. Tubes were incubated at 25 °C for one month to colonize the substrate with the fungus. After transferring the pre-germinated seedlings, tube were wrapped with aluminum foil to protect the roots from direct light. Plants were kept in a growth chamber and grown at 23±2 °C with 16 h photoperiod of 250 μmol photon m⁻² s⁻¹ light. After 4 months of growth period, shoot height, root length, seedling's fresh weight, and dry weight were measured to study the effect of different *Suillus* isolates on pine growth.

Growth, biomass and ectomycorrhizal root colonization

Shoot height, root length and fresh weights of seedlings were measured directly after washing the roots with tap water. To determine the seedlings dry weight, seedlings were oven dried at 70°C until constant weight was achieved. The percentage of ectomycorrhizal colonization (number of ECM short root/total number of short roots × 100%) in each root sample was determined visually under the stereomicroscope for each soil treatment.

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $p < 0.05$. All the analysis was performed using Graph Pad Prism 5.0 software.

RESULTS

Suillus isolates obtained from the Northwestern Himalayas

All of the *Suillus* isolates got isolated on malt extract (ME) agar (2% w/v) media. Although a few cultures were obtained also on Modified Melin-Norkrans (MMN) agar media, but the growth rate was comparatively low or otherwise poor. PDA (Potato dextrose agar) media was not found satisfactory for isolating *Suillus* cultures as we didn't get any culture isolated on PDA agar. Total of eight cultures were isolated from fresh basidiocarps (Table 1) and have been designated from "SNW01–SNW08" (SNW stands for '*Suillus* species from north western Himalayas'). The corresponding collection number and PUN number of basidiocarps from which cultures were isolated are also mentioned in Table 1.

Table 1. Different *Suillus* isolates (SNW01–SNW08) obtained from the basidiocarps collected from coniferous forests of the northwestern Himalayas, India

S. No.	Species	Collection/PUN No.	Isolate	MTCC accession No.
01	<i>Suillus triacicularis</i>	SHP27/PUN 5538	SNW01	11954
02	<i>Suillus indicus</i>	SHP07/PUN 6578	SNW02	11955
03	<i>Suillus himalayensis</i>	SHP26/PUN 5537	SNW03	11956
04	<i>Suillus granulates</i>	SJK13/PUN 5525	SNW04	11957
05	<i>Suillus sibiricus</i>	SJK01/PUN 5520	SNW05	11958
06	<i>Suillus sibiricus</i>	SHP05/PUN 6577	SNW06	11959
07	<i>Suillus sibiricus</i>	SHP12/PUN 6579	SNW07	11960
08	<i>Suillus sibiricus</i>	SUK12/PUN 5532	SNW08	11961

RFLP analysis of *Suillus* isolates

Variation within ITS region of all the *Suillus* isolates (SNW01, SNW02, SNW03, SNW04, SNW05, SNW06, SNW07 and SNW08) was examined by RFLP analysis of ITS-PCR products obtained with primers ITS1 and ITS4. PCR

products (approx. 700 bp) amplified from all the *Suillus* isolates were digested with the restriction endonucleases. Restriction digests produced using three different restriction enzymes *i.e.*, *AluI*, *HaeIII* and *MboI* are shown in Fig. 1. The restriction fragments obtained with all the three endonucleases were used to determine polymorphism among different isolates. Due to poor visibility in the gel, fragments below 61 bp were ignored during RFLP analysis. Restriction digestion with *AluI* resulted into two, *HaeIII* into four and *MboI* into three types of restriction patterns for all the isolates obtained in the present study. RFLP patterns of ITS region grouped the isolates into five different ITS-RFLP taxa. BlastN analysis also revealed that these isolates belonged to five different *Suillus* species.

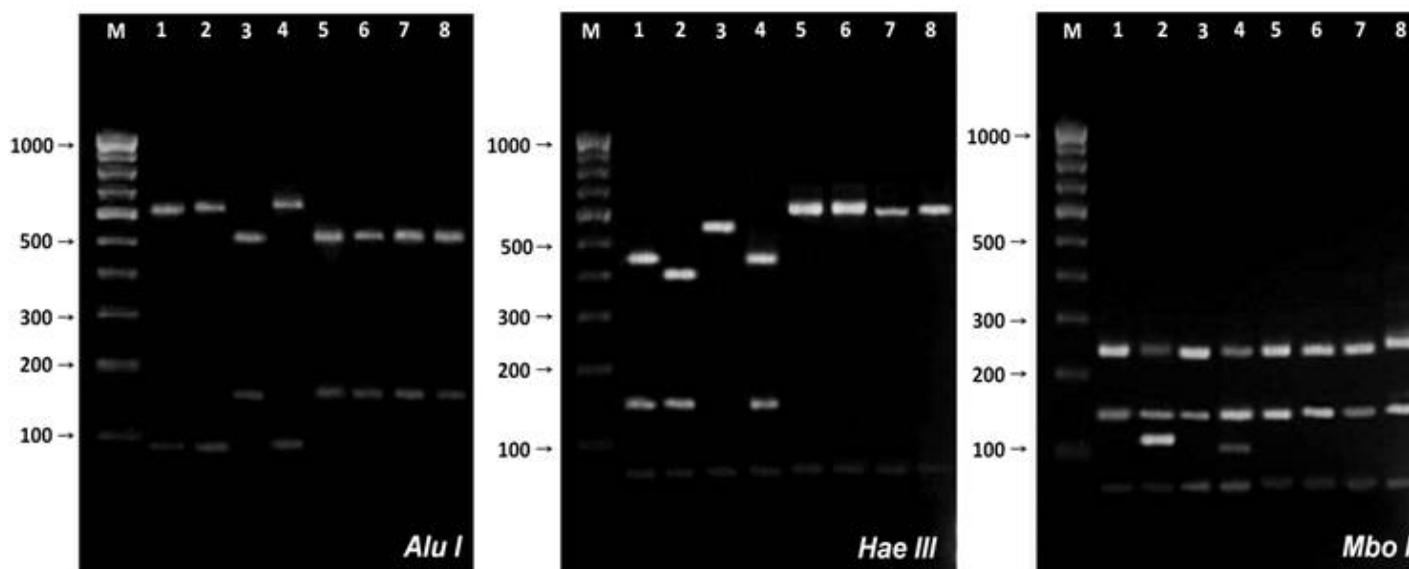


Figure 1. ITS-RFLP Analysis of *Suillus* isolates digested with three different restriction enzymes (*AluI*, *HaeIII*, and *MboI*). Lane M-DNA marker, Lane 1-8 are isolates of SHP27/SNW01, SHP07/SNW02, SHP26/SNW03, SJK13/SNW04, SJK01/SNW05, SHP05/SNW06, SHP12/SNW07 and SUK12/SNW08, respectively. The figure is a composite figure made from different gels.

Radial growth and biomass yield

Radial growth of *Suillus* isolates examined in the present study ranged from 2.2 ± 0.20 cm in *S. granulatus* SNW04 to 5.0 ± 0.40 cm in *S. indicus* SNW02 (Fig. 2). Biomass yield varied from 0.56 ± 0.09 mg/ml in *S. granulatus* SNW04 to 4.16 ± 0.31 mg/ml in *S. indicus* SNW02 (Fig. 3). *S. indicus* isolates SNW02 and *S. sibiricus* SNW06 showed comparatively higher radial growth than most of the other *Suillus* isolates and *S. granulatus* isolate SNW04 showed the lowest growth. Thus, significant inter-specific differences within the growth values of *Suillus* isolates were detected (Fig. 2, 3). On the basis of radial growth, *Suillus* isolates of present study can be divided into two types: one with high growth values (SNW01, SNW02, SNW06, SNW07 and SNW08) and other with low growth values (SNW03, SNW04 and SNW05). Also, significant intra-specific differences were detected in growth within the *S. sibiricus* isolates. *S. sibiricus* isolate SNW05 exhibited significantly lower radial growth and dry weight as compared to other *S. sibiricus* isolates, whereas *S. sibiricus* isolate SNW06 showed the highest value. Isolates of *S. sibiricus* (SNW06, SNW07 and SNW08) were more homogeneous in growth and no significant intra-specific differences in radial growth and dry weight were found within these strains. In general, *S. sibiricus* isolates can also be divided into two groups either with high growth values (SNW06, SNW07 and SNW08) or with low growth value (SNW05).

Mycorrhizal root colonization of *P. wallichiana* seedlings

All the *Suillus* isolates tested colonized the roots of *P. wallichiana* seedlings irrespective of their natural host type. Mycorrhizal root colonization of *P. wallichiana* roots by different isolates of *Suillus* species varied considerably (Fig. 4) and ranged from 23% in case of *S. triacicularis* SNW01 to 71% in *S. sibiricus* SNW06. Variations in the root colonization were also observed even within the isolates of *S. sibiricus* (isolate SNW05–SNW08) that were found to be 37, 71, 62 and 49% in

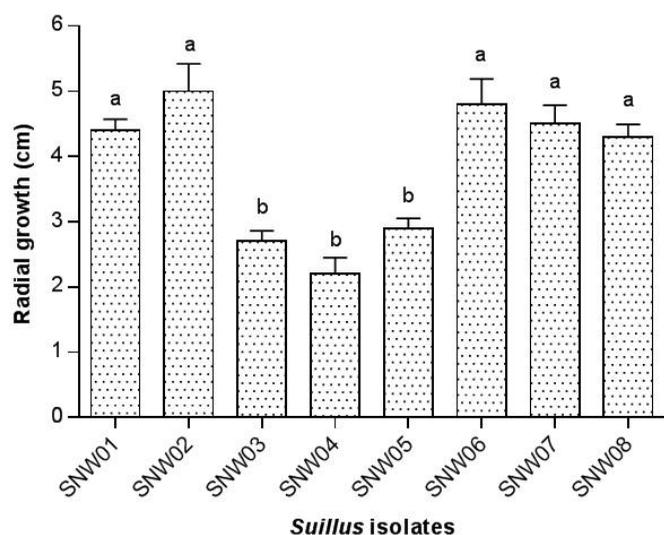


Figure 2. Radial growth (cm) of *Suillus* isolates (SNW01–SNW08) on 2% malt extract agar medium as inferred from means of colony diameter. Different letters represent significant differences among inoculation treatments according to the Tukey's test at $p < 0.05$

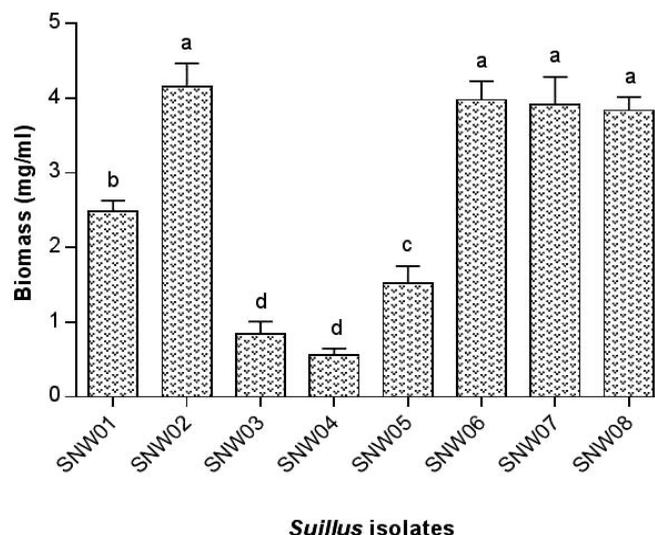


Figure 3. Biomass yield (mg/ml) of *Suillus* isolates (SNW01–SNW08) in 2% malt extract broth medium as inferred from means of mycelium dry weight. Different letters represent significant differences among inoculation treatments according to the Tukey's test at $p < 0.05$

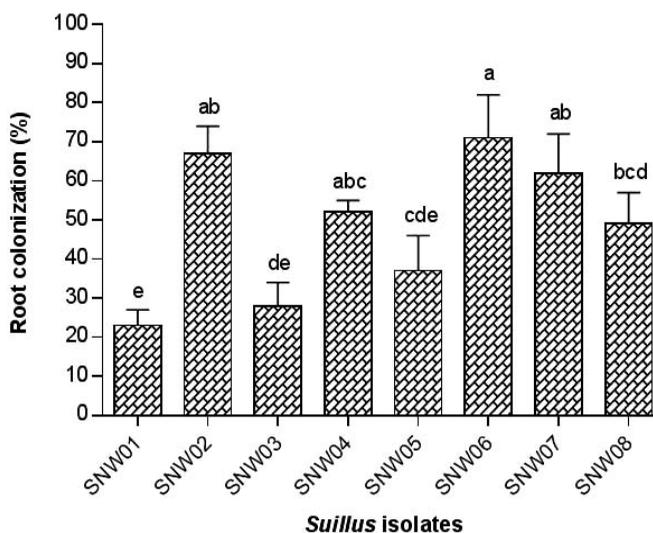


Figure 4. Effect of inoculation with different *Suillus* isolates on ectomycorrhizal root colonization of blue pine (*Pinus wallichiana*) seedlings. Different letters represent significant differences among inoculation treatments according to the Tukey's test at $p < 0.05$.

S. sibiricus SNW05, *S. sibiricus* SNW06, *S. sibiricus* SNW07 and *S. sibiricus* SNW08, respectively. Among the different *Suillus* isolates, treatment with *S. sibiricus* SNW06 (71%) showed significantly higher mycorrhizal root colonization followed by *S. indica* SNW02 (67%). On the contrary, *S. triacicularis* SNW01 (23%) showed significantly lower root colonization. Treatments with other isolates showed intermediate colonization percentage values. No ECM colonization was observed on un-inoculated control seedlings.

Influence on growth and biomass of *P. wallichiana* seedlings

Impact of all the *Suillus* isolates inoculations on growth and biomass of *P. wallichiana* seedlings were studied and outcomes are illustrated in Table 2. The results revealed that all the *Suillus* inoculants generally enhanced growth of *P. wallichiana* seedlings and significantly improved the plant growth (seedling height and root length) and biomass (fresh and dry plant

weight) compared to the un-inoculated control treatment. Among the different *Suillus* inoculants tested individually, the *S. sibiricus* isolate SNW06 showed significantly higher plant growth and biomass followed by *S. indicus* SNW02, *S. sibiricus* SNW07 and *S. granulatus* SNW04 isolates. The blue pine seedlings inoculated with *S. sibiricus* SNW06 showed 51.39, 66.67, 43.57 and 52.29 percent increase in shoot height, root length, plant fresh and dry weight, respectively as compared to control treatment. The highest growth enhancement by *S. sibiricus* SNW06 may be attributed to higher mycorrhizal colonization (71%) of *P. wallichiana* roots by *S. sibiricus* SNW06 isolate in comparison to the other isolates. The minimum increases in the growth and biomass was observed in case of *S. himalayensis* SNW03, which comparatively exhibits poor mycorrhizal root colonization (28%). Thus, the present study identified *S. sibiricus* SNW06 and *S. indicus* SNW02 as efficient *Suillus* isolates for enhancement of growth and biomass of blue pine seedlings.

Table 2. Influence of inoculation with different *Suillus* isolates on growth and biomass of blue pine (*Pinus wallichiana*) seedlings after four months of the growth period

<i>Suillus</i> isolate	Shoot height (cm)	Root length (cm)	Plant fresh weight (mg/plant)	Plant dry weight (mg/plant)
SNW01	8.2±0.6 (ab)*	8.6±1.5 (ab)	579±91 (abc)	268±28 (ab)
SNW02	10.8±1.5 (a)	11.2±2.1 (a)	727±77 (ab)	329±31 (a)
SNW03	7.7±0.8 (ab)	7.8±0.6 (ab)	557±28 (bc)	251±29 (ab)
SNW04	9.3±1.4 (ab)	9.8±1.6 (ab)	686±84 (abc)	302±44 (ab)
SNW05	8.4±1.7 (ab)	8.9±0.5 (ab)	590±43 (abc)	276±61 (ab)
SNW06	10.9±1.1 (a)	11.5±1.9 (a)	748±62 (a)	332±49 (a)
SNW07	9.9±1.9 (ab)	11.0±1.6 (a)	712±38 (ab)	315±17 (ab)
SNW08	8.7±1.0 (ab)	9.1±0.6 (ab)	652±73 (abc)	297±41 (ab)
Control	7.2±0.9 (b)	6.9±1.4 (b)	521±52 (c)	218±35 (b)

* Letters within parenthesis in a single column denote significant differences among the treatments by the Tukey's test at $p < 0.05$. Mean±SD with the same small letters are not significantly different at $p = 0.05$.

DISCUSSION

Molecular ecological studies on ECM fungi have mainly employed restriction analysis of the ITS region (ITS-RFLP) for identification and differentiation at the species level [29]. ITS-RFLP analysis of *Suillus* isolates of present study (Fig. 1) grouped them into five different ITS-RFLP taxa. Molecular typing using ITS-RFLP technique detected twelve ITS-RFLP taxa on non-inoculated, *S. collinitus*-inoculated, and naturally regenerated trees in a fire-disturbed *P. halepensis* plantation [30]. In naturally established *Pinus nigra* nursery, RFLP patterns of ITS region resulted in typing of four ITS-RFLP taxa [31]. Beatriz *et al.* (2006) molecularly characterized nineteen *Suillus* isolates from different pine forests of Central Spain and based on ITS-RFLP analysis, clustered the isolates into six different groups [14]. These studies discussed herein confirm the potential of ITS-RFLP for the identification, molecular characterization, species delimitation and ecological studies of *Suillus* species.

Significant inter-specific differences were found in radial growth and mycelia dry weight of *Suillus* isolates when grown under *in vitro* conditions (Fig. 2 & Fig. 3). Five *Suillus* isolates (*S. triacicularis* SNW01, *S. indicus* SNW02, *S. sibiricus* SNW06, *S. sibiricus* SNW07 and *S. sibiricus* SNW08) showed significantly higher radial growth as compared to other three *Suillus* isolates (*S. himalayensis* SNW03, *S. granulatus* SNW04 and *S. sibiricus* SNW05) that showed significantly lower radial growth. Considering the mycelia dry weight, four *Suillus* isolates (*S. indicus* SNW02, *S. sibiricus* SNW06, *S. sibiricus* SNW07 and *S. sibiricus* SNW08) exhibited highest growth values, one (*S. triacicularis* SNW01) high-intermediate value, one (*S. sibiricus* SNW05) intermediate value and rest two (*S. himalayensis* SNW03 and *S. granulatus* SNW04) showed lowest growth values. Moreover, significant intra-specific differences were also detected within radial growth and mycelia dry weight of different *S. sibiricus* isolates. Furthermore, *in vitro* mycorrhizal root

colonization of *P. wallichiana* roots by *Suillus* isolates was studied. The results revealed that *Suillus* isolates differ in their ability to colonize *P. wallichiana* roots and variations were also observed even within the isolates of *S. sibiricus* (Fig. 4). Inter-specific as well as intra-specific variations in axenic fungal growth and *in vitro* mycorrhizal root coonization of *P. halepensis* roots by different *Suillus* isolates have also been detected by Beatriz et al. (2006) from Central Spain [14].

Outcomes of the present investigation suggested that all the *Suillus* isolates tends to increase seedlings growth (seedlings height, root length) and biomass (fresh weight and dry weight) as compared to the un-inoculated control seedlings. Following ectomycorrhizal inoculations, increase in growth and nutrients content of pine seedlings have been also observed by various authors while working with a variety of pines and ECM fungi [5-10, 14]. While contemplating *in vitro* mycorrhizal capacity of different indigenous *Suillus* isolates for the growth of *P. halepensis* seedlings, Beatriz et al. 2006 noticed that *Suillus* isolates stimulated the growth of *P. halepensis* seedlings in different rates [14]. Our experiments also showed the similar results. In the present study, *S. sibiricus* isolate SNW06 showed significantly higher plant growth and biomass in comparison to other *Suillus* isolates. The enhancement in the growth of blue pine seedlings was followed by *S. indicus* SNW02, *S. sibiricus* SNW07 and *S. granulatus* SNW04 isolates. Comparatively lower increase in the growth and biomass of blue pine seedlings was detected when inoculated with *S. himalayensis* SNW03. On the basis of mycorrhizal root colonization and the effects on growth and biomass of blue pine seedlings, *S. sibiricus* SNW06 and *S. indica* SNW02 were found to be the most effective and suitable *Suillus* isolates for growth of *P. wallichiana* seedlings and therefore suggested suitable for mass inoculums production and field inoculations.

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