Diversity of *Suillus* species associated with conifer trees in North Western Himalayan region of India

A Thesis
Submitted in fulfillment of the requirements for
the award of degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

Submitted by
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CERTIFICATE

This is to certify that the thesis entitled **"Diversity of *Suillus* species associated with conifer trees in North Western Himalayan region of India"** is submitted by Mr. Balwant Verma, in fulfillment of the requirements for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology, Thapar University, Patiala, Punjab, India and contains a record of the candidate's own independent and original research work carried out by him under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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DECLARATION

I hereby declare that the work which is being presented in this thesis "**Diversity of *Suillus* species associated with conifer trees in North Western Himalayan region of India**" for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology, Thapar University, Patiala is true and original record of my own independent and original research work carried out under the supervision of Dr. M. Sudhakara Reddy, Professor, Department of Biotechnology, Thapar University, Patiala, Punjab, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

(Balwant Verma)

Dedicated to my Adored Parents ....

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(Balwant Verma)

List of publications

The following publications are outcome of the present research work:

1. **Balwant Verma** and M. Sudhakara Reddy. 2014. *Suillus triacicularis* sp. nov., a new species associated with *Pinus roxburghii* from northwestern Himalayas, India. *Phytotaxa*, 162 (3): 157–64.
2. **Balwant Verma** and M. Sudhakara Reddy. 2014. *Suillus himalayensis (Basidiomycota, Agaricomycetes,* Boletales), a new species associated with *Pinus wallichiana* from the northwestern Himalayas, India. *Nova Hedwigia*, 99 (3–4): 541–550.
3. **Balwant Verma** and M. Sudhakara Reddy. 2014. *Suillus indicus* sp. nov. (Boletales, Basidiomycota), a new boletoid fungus from northwestern Himalayas, India. *Mycology: An International Journal of Fungal Biology*, DOI: 10.1080/21501203.2014.988770.

Abstract

The diversity of the genus *Suillus* has not been explored thoroughly from the northwestern Himalayan region of India. By virtue of this, several basidiocarps of the genus *Suillus* were collected from different parts of this region and characterized morphologically as well as molecularly. The present research work described 7 species of *Suillus*. Among these, 3 taxa namely, *S. triacicularis* sp. nov., *S. indicus* sp. nov., and *S. himalayensis* sp. nov., were new to the science while *S. flavidus* and *S. placidus* were new records from India. Attempts were also made to isolate the cultures from each basidiocarps collected. In total, 8 *Suillus* isolates belonging to five different species (*S. triacicularis* sp. nov., *S. indicus* sp. nov., *S. himalayensis* sp. nov., *S. granulatus* and *S. sibiricus*) were obtained from basidiocarps. All these *Suillus* isolates exhibited inter-specific as well as intra-specific variations in axenic fungal growth, extracellular enzyme activities, and *in vitro* mycorrhizal capacities with *Pinus wallichiana* seedlings. On the basis of fungal growth, enzyme activities, mycorrhizal colonization, and the effects on seedlings growth, biomass and nutrients content; *S. sibiricus* SNW06 and *S. indicus* SNW02 were found to be the most effective and suitable *Suillus* isolates for growth promotion of *P. wallichiana* seedlings and therefore selected for mass inoculum production. Optimization of various parameters for mass inoculum production of both the *Suillus* isolates resulted in a significant increase in the radial growth and biomass yield of both the selected *Suillus* isolates. Further, the effects of both the selected *Suillus* isolates and biochar amendment on *P. wallichiana* growth and rhizosphere properties were evaluated. Based upon the present investigation, *S. sibiricus* SNW06 was found to be an efficient mycorrhizal strain as compared to *S. indicus* SNW02 and therefore, recommended for the production of mycorrhizal *P. wallichiana* seedlings in forestry practices. Also biochar application to the soil, especially along with mycorrhizal inoculations, was found to be advantageous for improvement of growth and nutrients content of *P. wallichiana* seedlings as well as the soil physico-chemical properties and enzyme activities.

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Abbreviations

A	Absorbance
ACPase	Acid phosphatase
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of Variance
BC	Biochar
BI	Bayesian inference
BLAST	Basic local alignment search tool
bp	Base pair
BPP	Bayesian posterior probabilities
C	Carbon
°C	Degree celsius
Ca	Calcium
$CaCl_2$	Calcium chloride
Cd	Cadmium
CEC	Cation exchange capacity
cm	Centimeter(s)
cm^3	Cubic centimeter
C/N	Carbon nitrogen ratio
CTAB	Cetyl trimethylammonium bromide
Cu	Copper
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
dNTP	2’-deoxynucleotide-5’-triphosphate
ECM	Ectomycorrhizal
EDTA	Ethylenediamine-tetra acetic acid
$FeSO_4$	Ferrous sulphate
Fig.	Figure
g	Gram
h	Hour(s)
ha	Hectare
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide
IGS	Intergenic spacer
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS	Internal transcribed spacer
K	Potassium
kg	Kilogram
KOH	Potassium hydroxide
l	Litre
LB	Luria-Bertani
L/W	Length/width
M	Molar
m	Metre(s)
ME	Malt extract
Mg	Magnesium

$MgCl_2$	Magnesium chloride
µg	Microgram
µl	Microlitre(s)
µM	Micromolar
µm	Micrometer(s)
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mM	Millimolar(s)
mm	Millimetre(s)
MMN	Modified Melin-Norkans medium
mtDNA	Mitochodrial DNA
MUB	Modified universal buffer
N	Nitrogen
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
ng	Nanogram
NH_3	Ammonia
NH_4^+	Ammonium ion
NH_4OH	Ammonium hydroxide
nLSU	Nuclear ribosomal large subunit
nm	Nanometer
NO_2^-	Nitrite ion
NO_3^-	Nitrate ion
nSSU	Nuclear ribosomal small subunit
OD	Optical density
OM	Optimized medium
P	Phosphorus
PCR	Polymerase chain reaction
PDA	Potato dextrose agar medium
PEG	Polyethylene glycol
%	Percent
pH	Potential of hydrogen
Pi	Inorganic phosphate
pNP	p-nitrophenol
ppm	Parts per million
PUE	Protease unit equivalents
Q	Quotient
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
S	Sulphur
s	Second(s)
sp. nov.	Species novum
sq km	Square kilometer
TBE	Tris/Borate/EDTA

TE	Tris-EDTA
TOC	Total organic carbon
TPF	1, 3, 5-triphenyl tetrazolium formazan
Tris	Tris-(hydroxymethyl-) aminomethane
U	Unit
UV	Ultraviolet
V	Volts
v/v	Volume by volume
v/v/v	Volume by volume by volume
w/v	Weight by volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
ybp	Years before present
Zn	Zinc

Chapter 1

Introduction

The mutualistic associations between plants and fungi are called ‘mycorrhizas’. In the modern world, about 80% of the plant species and 92% of the plant families studied are characteristically mycorrhizal (Wang and Qiu 2006). It has been speculated that plants have colonized land and moved from aquatic to aerial habitat possibly because of the mycorrhizal fungi (Harley and Harley 1987; Selosse and Le Tacon 1998; Read et al. 2000; Brundrett 2002; Courty et al. 2010a). In well fertilized soils, where nutrient and water supply are not the limiting factor, plants can grow well and develop efficiently without the fungal partners. However, in several terrestrial habitats with limited water supply and the scarcity of soluble minerals, they play a pivotal role in development and stability of host plant and therefore considered as ‘ecologically obligate’ (Read 1993). The mycorrhizal fungi are best adapted to explore the below ground resources, especially the non-soluble mineral elements (Martin 1985; Chalot and Bran 1998; Buscot et al. 2000; Smith and Read 2008). They benefit plants in a number of ways mainly by enhancing plant nutrients uptake, particularly nitrogen and phosphorus, and by improving plant water uptake in low water environments. On the other hand, the plants are well adapted to gaseous exchange and photon collection, thus exploiting the atmospheric resources. They provide vital carbohydrates and important vitamins to the non-photosynthetic fungal symbiont through the root interface. Thus, both the symbionts of mycorrhizas form mutualistic relationship and exhibit complementary adaptations to live on terrestrial conditions.

Mycorrhizal fungi are symbiotic root colonizing fungi, which become integrated to the physical structure of plant roots. Based on the types of mycorrhizal structures formed by the mycorrhizal fungi on plant roots, the mycorrhizas can be broadly classified into ectomycorrhizas, ecto-endomycorrhizas, and endomycorrhizas (Wilcox 1991). Ectomycorrhizal (ECM) fungi are septate fungi, which form a sheath around the root and extend a network of external mycelium into the soil (Fig. 1.1). They grow between the root cortical cells, without protruding the individual cells, to form the Hartig net. Unlike ECM fungi, endomycorrhizal fungi penetrate the root cell walls to form intracellular arbuscules (arbuscular mycorrhizas) or coils (ericoid and orchid mycorrhizas) and grow mostly within the plant roots with few external hyphe (Fig. 1.1). Ecto-endomycorrhizas display an intermediate form, which is generally characterized by a sheathing structure, a rudimentary Hartig net and intracellular structures such as pegs (monotropoid mycorrhizas) or coils (arbutoid mycorrhizas) (Smith and Read 1997). Among these different types of mycorrhizal symbioses, arbuscular mycorrhizas that appeared about 400–460 million years ago (Simon et al. 1993; Remy et al. 1994) are suggested to be the ancestral (Selosse and LeTacon 1998; Heckman et al. 2001; Wang and Qiu 2006; Courty et al. 2010a) and most dominant mycorrhizal symbioses involved with about 85% of all the plant families (Courty et al. 2010a). Ectomycorrhizas are more recently evolved form of mycorrhizal symbioses involved with about 10% of the plant families (Courty et al. 2010a) and supposed to be originated between 220 and 150 million years ago (Bruns and Shefferson 2004; Alexander 2006).

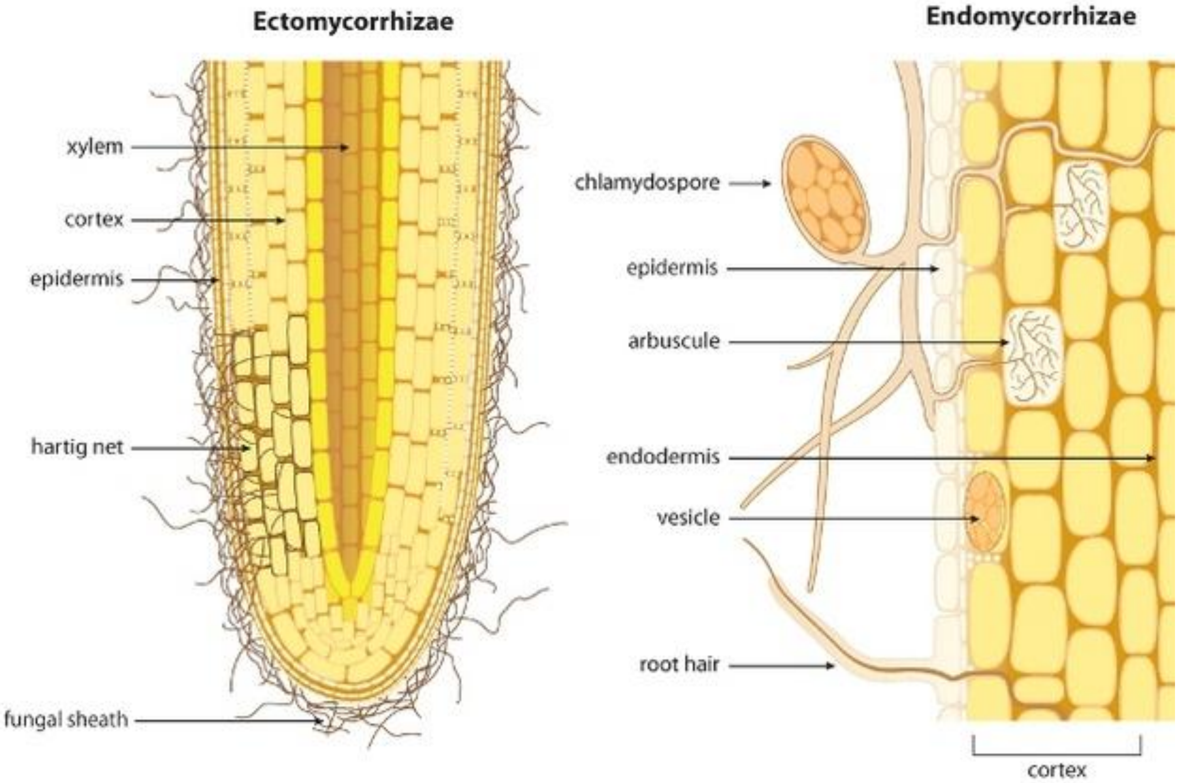


Fig 1.1 Schematic diagram showing difference between ectomycorrhizae and endomycorrhizae colonization of plant roots (Adapted from: McNear Jr., David H. © 2013, Nature Education Knowledge)

Ectomycorrhizas include a diverse group of organisms, with about 5000-6000 species belonging to several families found mainly in the phyla Basidiomycota and Ascomycota (Molina et al. 1992; Bentivenga 1998). Communities of ECM fungi are the dominant mycorrhizal symbionts in the boreal, temperate, and Mediterranean forest ecosystems and are also found in some tropical rain forest environments. In these diverse plant biomes, they are associated with trees belonging to the families *Abietaceae*, *Betulaceae*, *Dipterocarpaceae*, *Fagaceae*, *Myrtaceae*, *Pinaceae*, *Salicaceae*, *Tiliaceae* and some of the *Rosaceae*. Fungal auxin produced by ectomycorrhizal fungi is involved in the formation of ectomycorrhizas (Gay et al. 1995). The auxin-overproducing mutants of the ECM fungus *Hebeloma cylindrosporum* (Durand et al. 1992) have been shown to have increased mycorrhizal activity (Gay et al.

1994). In nature, ECM fungi improve the health and growth of forest trees and benefit them in number of ways. ECM fungi are well adapted to mobilize the sparse heterogeneous resources (Read 1993) and extend a network of external mycelium into the soil resulting in three-dimensional exploration of the substrates. The ectomycorrhizal symbioses allow plants to increase nutrients uptake, particularly in nutrient poor environments, (Martin 1985; Del Vecchio et al. 1993; Chalot and Bran 1998; Smith and Read 2008) and exploit the nutrient pools that would be unavailable to a non-mycorrhizal plant. They are also beneficial to the host plant under drought conditions and assist with water uptake (Smith and Read 1997). In addition, ECM fungi are able to protect plants against pathogens (Smith and Read 1997) and toxic elements in the environment, such as trace metals (Colpaert and Van Assche 1993; Jentschke and Godbold 2000; Adriaensen et al. 2004, 2005; Courbot et al. 2004; Krznaric et al. 2009). Moreover, the tips of ectomycorrhizal plant roots are also capable of forming mycorrhizas with ericoid mycorrhizal fungi (Bergero et al. 2000). Thus, ECM fungi positively affect forest ecosystems by enhancing nutrients and water uptake, increasing the resistance of host trees against water shortage and high concentration of heavy metals, and protecting roots from soil pathogens. This is reciprocated by the allocation of vital carbohydrates such as glucose and important vitamins such as biotin and thiamine by the plant to the fungal symbiont.

Suillus Gray is one of the most prominent genera of ECM fungi in the order *Boletales*. The genus was described by S.F. Gray in 1821 (Gray 1821) and belongs to the family *Suillaceae*, order *Boletales*, class *Agaricomycetes* and phylum *Basidiomycota*. Around 50 different species of *Suillus* have been reported throughout the world so far (Kirk et al. 2008). The pioneer work on documenting *Suillus* diversity was done by Smith and Thiers (1964). Thereafter, several other investigations on diversity and evolutionary phylogenetics of *Suillus* species from different parts of the world have been reported (Corner 1972; Thiers 1979; Kretzer et al. 1996; Kretzer and Bruns 1997; Wu et al. 2000; Manian et al. 2001; Beatriz et al. 2006; Feng et al. 2008; Bruns et al. 2010; Sarwar and Khalid 2014). Very little is known about *Suillus* diversity from the Himalayan region of India. *Suillus sibiricus* (Singer) and *Suillus granulatus* (L.) Roussel have been reported from Himachal Pradesh and Jammu & Kashmir states of India, respectively (Sagar and Lakhanpal 2005, Dar et al. 2010). *Suillus* species are important ectomycorrhizal symbionts in temperate, boreal and Mediterranean ecosystems (Singer 1986; Kretzer et al. 1996; Wu et al. 2000). In these ecosystems, they exhibit a high degree of host specificity toward members of the family *Pinaceae* (Smith and Thiers 1964; Kretzer et al. 1996; Wu et al. 2000) and coexist with the natural occurrence of *Pinaceae*. Generally, they are found in northern hemisphere although a few species have been introduced into the southern hemisphere along with pines (Dunstan et al. 1998). Excursions for ECM diversity in the Mediterranean pine forests have shown that *Suillus* sporocarps are among the abundant and dominant ECM fungal species (Beatriz et al. 2006; Rincon et al. 2006).

In India, the northwestern Himalayan region is a natural abode of large number of conifer plants, which mainly includes *Pinus wallichiana*, *Pinus roxburghii*, *Pinus girardiana*, *Cedrus deodara*, *Abies pindrow* and *Picea smithiana*. The variations in topographical features along longitude, latitude and altitude create climatic variations resulting in unique and rich biodiversity elements (Samant et al. 1998). Most of the region is covered by snow Mountains, glaciers of higher Himalayas and dense forest covers of mid-Himalayas. Forests are one of the major natural resources of the northwestern Himalayas. The region serves as a rich repository of plant wealth in diverse ecological systems. Due to varying altitudinal ranges, climate of the region is favorable for growth of a large variety of plants that ranges from subtropical to dry temperate alpine zone. On the basis of interpretation of satellite data, the forest cover of the northwestern Himalayan region is spread over an area of 61,714 sq km and constitutes approximately 18.62% of the total geographical area of the region (India State of Forest Report 2011).

The forest resources present in the northwestern Himalayan region are an integral part of the livelihood of local communities. Communities residing in this region are dependent upon these forest resources for meeting with routine livelihood needs. Apart from their usage as timber, plants are also used as fuel, medicine, food, fodder, agricultural tools, spiritual needs and various other purposes by indigenous societies. Average annual production of timber and fuel wood from forest resources of the northwestern Himalayas has been estimated to be 537,000 cubic meter and 70,050 tons, respectively (Kashyap et al. 2014). The forest cover also plays a major role in maintaining the hydrological regime of the region as well as the perennial rivers throughout the year, which also feed the adjoining plain areas for agricultural production. It also maintains optimum temperature for fruits and off-season agricultural practices and conservation of biodiversity in the region. Therefore, sustaining forestry in the region also means conservation of biodiversity, water resources and protection of inhabitant’s interests. Global climatic changes, such as global temperature rise, shrinking ice sheets, glacial retreat, declining arctic sea ice, sea level rise, ocean acidification etc., human activities, forest fires and several other natural cataclysms have marked impacts on forest ecosystems. These factors not only influence the forest vegetation but also the below ground micro flora, especially the ECM fungi, which are generally overlooked. In fact, ECM fungi are among the most critical and essential components of the forest ecosystems that need to be documented and conserved. Several species of ECM fungi have been reported to be declining, threatened or endangered (Arnolds and Kuyper 1996).

The survival, establishment and growth of out-planted seedlings predominantly rely upon the ECM fungi associated with their root systems (Perry et al. 1987; Kropp and Langlois 1990; Stenström et al. 1990; Le Tacon et al. 1992; Pera et al. 1999; Baum et al. 2002; Dunabeitia et al. 2004). Most of the forestry practices commonly use uninoculated seedlings grown in nurseries for out-planting purposes in the forests. These uninoculated seedlings are poorly colonized by ECM fungi (Dunabeitia et al. 2004; Menkis et al. 2005) and hypothesized to be incompetent for afforestation programmes due to the absence of mycorrhizas (Bjorkman 1970; Mikola 1970; Marx 1980). This problem can be overcome by using pre-inoculated seedlings with selected indigenous ECM fungi (Cordell et al. 1987; Le Tacon et al. 1992; Brundrett et al. 1996) that are best adapted to the local environmental conditions (Garbaye 1984; Perry et al. 1987). Therefore for afforestation of a defined region, it is of great significance to collect, identify, isolate and evaluate the indigenous ECM fungi to select effective isolates for successful establishment of out-planted mycorrhizal seedlings. Pine species are an extensively used plant species for afforestation programmes due to their traditional, medicinal, and industrial utilities. Considering the commercial plantations and afforestation of pine species, the ectomycorrhizal community associated with the pine is also of utmost importance and needs to be documented and conserved. In the present study, *Suillus* species were selected due to their remarkable specificity towards

Pinaceae. Moreover, the mycelia of *Suillus* species are easy to culture as compared to the other ECM fungi and this advantage has been exploited in different studies (Wu et al. 2000; Manian et al. 2001; Beatriz et al. 2006; Rincon et al. 2006). Furthermore, many authors have shown that *Suillus* species improve the plant growth, survival, mineral nutrition, (Timonen et al. 1996, 1997; Wallander 2000; González-Ochoa et al. 2003; Beatriz et al. 2006; El Karkouri et al. 2006) and also provide tolerance against the heavy metal toxicity in pine seedlings (Colpaert and Van Assche 1993; Adriaensen et al. 2004, 2005; Krznaric et al. 2009).

Though the afforestation of pine trees is essential, they also brought problems associated with pine trees bio-waste, especially the pine needles spread over the forest floor. Dried-up pine needles are highly inflammable and catches fire easily that may spread across the entire forest area causing damage to forest wealth as well as to the surrounding areas. In the past few decades, forest wealth worth several millions has been damaged in the northwestern Himalayan region due to forest fires. These fallen pine needles also prevent the water absorption by the soil, which may further cause depletion in ground water table. Additionally, the dry pine foliage almost entirely covers the forest floor and inhibits the growth of other forest vegetation. As a short term procedure, utilization of pine needles for biochar production can outsource the work of removing the pine needles from pine forests. Biochar is produced from biomass by the pyrolysis of biomass feedstock and is predominantly composed of recalcitrant organic carbon. Numerous studies have emphasized the importance of biochar as soil additive in long term storage of carbon in soils, improvement of physico-chemical properties of soil as well as the overall increase in plant growth and crop yields (Glaser et al. 2002; Lehmann et al. 2002, 2005, 2006; Glaser and Woods 2004; Day et al. 2005; Rondon et al. 2005, 2007; Lehmann and Rondon 2006; Marris 2006; Fowles 2007; Glaser 2007; Blackwell et al. 2009; Chan and Xu 2009; Downie et al. 2009). Moreover, the biochar application to the soil can also increase mycorrhizal colonization of the plant roots (Harvey et al. 1976; Saito 1990; Ishii and Kodoya 1994, 2007; Mori and Marjenah 1994; Ezawa et al. 2002; Matsubara et al. 2002; Yamato et al. 2006; Rillig et al. 2010). Thus, pine needle derived biochar can be used as soil amendment in pine nurseries to improve pine growth and mycorrhizal symbiosis. This will led to removal of pine needles from forest floor, whereas biochar produced can be used for growth enhancement of pine seedlings and successful tree establishment in the field.

Gaps in study

Suillus species are among the abundant and persistent ECM fungi found in the northwestern Himalayas of India. Considering the critical role of ECM fungi in plant growth, water uptake, mineral nutrition and survival of plants in metal contaminated soil, it is of utmost important to study their diversity. Although few reports are available (Dar et al. 2010; Sagar and Lakhanpal 2005), the diversity of the genus *Suillus* has not been explored so far from this region. Further, stress is imposed by reports of ECM declining, which is of major concern while considering the forest ecosystem. Besides documenting the diversity, it is of great significance to conserve the biodiversity (Atri and Lakhanpal 2002), which can be achieved by isolating and preserving the *Suillus* isolates. In context of India, there is no deposition of *Suillus* isolates in any internationally recognized culture bank. Moreover, the abilities of indigenous *Suillus* species, except for *S. granulatus* (Dar et al. 2010), and biochar additions to promote growth of *P. wallichiana* A.B. Jacks. have not been evaluated. These lacunae in the study fortify the essence of describing *Suillus* species of the northwestern Himalayan region of India and evaluation of their mycorrhizal capacity for plant growth promotion along with biochar amendment.

Aim and Objectives

The present study was aimed at determining the genetic diversity and interrelationships among the common northwestern Himalayan *Suillus* species based on the ribosomal DNA sequences and classical methods with a view to assess the potential of this region for species delimitation and diagnosis of the genus *Suillus* to species level. As a further step to characterize different *Suillus* isolates, their growth, root colonizing ability, extracellular enzyme activities and *in vitro* mycorrhizal capacity were studied. In addition, the benefits of selected *Suillus* isolates for the growth of a typical Himalayan conifer plant, i.e. *P. wallichiana*, with or without biochar addition were determined. The overall aim of such analysis was to document the diversity of *Suillus* species from the northwestern Himalayas and select isolates that are best adapted to the environmental conditions prevailing in this specific geographic region with aim to produce fungal inocula for afforestation programmes.

Keeping in view the paucity of our present knowledge on *Suillus* diversity from the northwestern Himalayas and the importance of *Suillus* species and biochar addition in plant growth, survival, mineral nutrition and metal amelioration, the present research work was undertaken with the following objectives:

1) To document the diversity of *Suillus* species from the northwestern Himalayan region of India

2) Physiological characterization of *Suillus* species to select suitable isolates for mass inoculum production

3) To optimize culture conditions for mass inoculum production of selected *Suillus* isolates

4) To study the influence of selected *Suillus* isolates and biochar amendment on plant growth and rhizosphere properties

Chapter 2

Reappraisal of Literature

The genus *Suillus* Gray was described by Samuel Frederick Gray with *Suillus luteus* (L.) Roussel as type species (Gray 1821). Later, the genus has been emended by Smith and Thiers (1964). They are commonly known as "slippery jacks" because of the pileus being slimy especially under wet conditions. The genus epithet is derived from the Latin word “sus”, which means "pig". The genus is generally characterized by viscid, slimy and readily decaying pileus, usually with sudecurrent to decurrent tubular hymenophore and central to excentric stipe often with conspicuous punctuate or glandular dots on its surface. Spore prints vary from light brown through yellow brown to dark chocolate brown and finally to olive shades. Microscopically, clustered cystidial elements usually occur in fascicles and contain colored amorphous pigments around the base of bundles or over the cystidia themselves. Generally, clamp connections are absent from the hyphae of the *Suillus* sporocarps. Mostly they form mycorrhizas with members of family the *Pinaceae* (particularly *Pinus*, *Larix* and *Pseudotsuga*), but a few species are also found associated with hardwoods. Main distinguishing inter-specific characters for species delimitations are color and ornamentation of the pileal surface, color and color change of flesh, tubes, pores and stipe, the presence/absence of a partial veil or annulus, pore shape and distribution, host, as well as the habitat. All *Suillus* species are edible, but many consider them undesirable due to their slimy cap cuticle. Removal of slimy cap cuticle and tube layers is highly advised before their consumption (Weber and Smith 1980). Their odor is mild and taste is mild or slightly acidic. There are about 30 edible *Suillus* species known worldwide (Boa 2012). Typical example of edible *Suillus* is *S. brevipes* (Orr and Orr 1979), which contains good amount of lipid content mainly linoleic acid, oleic acid and palmitic acid (Sumner 1973).

2.1 Systematic position of *Suillus*

Suillus is a genus of basidiomycete fungi belonging to the family *Suillaceae* and order *Boletales*. Before 1997, the genus *Suillus* was placed under the family *Boletaceae* of order *Boletales*. There is relatively extensive literature documenting the systematic and taxonomic organization of the order *Boletales* using morpho-anatomical characteristics (Singer 1986; Agerer 1999), pigment chemistry (Gill and Steglich 1987; Besl and Bresinsky 1997), and the sequence analysis (Bresinsky et al. 1999; Kretzer and Bruns 1999) of the basidiocarps. These findings showed remarkable variance in morpho-anatomy, hymenophores and pigment composition of basidiocarps within the *Boletales*. As a result, numerous new families and genera have been generated.

Scientific Classification (Kirk et al. 2008)**:**

Kingdom: Fungi
Subkingdom: Dikarya
Phylum: Basidiomycota
Class: Agaricomycetes
Order: Boletales
Family: *Suillaceae*
Genus: *Suillus*

On the basis of chemotaxonomic findings, *Suillus* species were found to be closely related to the *Gomphidiaceae* and *Rhizopogonaceae* as compared to the remaining *Boletales* (Besl and Bresinsky 1997). These results led to the formation of a new suborder *Suillineae* within the *Boletales*, which included *Gomphidiaceae*, *Suillaceae*, and *Rhizopogonaceae*. Thus, the genus *Suillus* was carved out from the family *Boletaceae* and now placed in the newly formed family *Suillaceae* along with the genera *Psiloboletinus* and *Truncocolumella*.

2.2 Diversity

About 50 species of *Suillus* have been reported from different parts of the world (Kirk et al. 2008). Among them majority of the species are described by Smith and Thiers (1964, 1971), Corner (1972) and Thiers (1976, 1979). Thereafter, several other investigations on diversity and evolutionary phylogenetics of *Suillus* species from different parts of the world have been reported (Kretzer et al. 1996; Kretzer and Bruns 1997; Wu et al. 2000; Manian et al. 2001; Beatriz et al. 2006; Feng et al. 2008; Bruns et al. 2010; Min et al. 2014; Sarwar and Khalid 2014). Kretzer et al. (1996) derived the ITS sequences from 47 isolates belonging to 38 recognized species of the genus *Suillus* from America, Canada, Europe and Asia. They also revealed that the generic and species concepts of *Suillus*, *GastroSuillus*, *Boletinus* and *Fuscoboletinus* should be reevaluated and suggested the collapsing of genera *Boletinus* and *Fuscoboletinus* into the genus *Suillus*. Later on, the genus *Gastrosuillus* was also collapsed into *Suillus* on the basis of ITS data (Kretzer and Bruns 1997). The phylogenetic relationship between fourteen eastern Asian and twenty two eastern North American S*uillus* species has been determined by Wu et al. (2000) and interrelationship among thirty four common European *Suillus* isolates representing eight species was studied by Manian et al. (2001). Moreover, nineteen different isolates representing seven species were recorded from Mediterranean area of central Spain (Beatriz et al. 2006). Also, the molecular identification and genetic diversity of twenty seven *Suillus* strains isolated from Inner Mongolia have been studied (Feng et al. 2008).

A new *Suillus* species, *S. quiescens* T.D. Bruns & Vellinga, was described from California and Oregon by Bruns et al. (2010). Recently, a world-wide key to the genus *Suillus* was given by Klofac (2013). There are very few reports of this particular genus from India. *Suillus brevipes*, *Suillus pallidiceps*, *Suillus punctatipes* and *Suillus subluteus* were reported from *Pinus patula* forests of Tamilnadu, India (Natarajan and Raman 1983). *Suillus sibiricus* (Sagar and Lakhanpal 2005) and *Suillus granulatus* (Dar et al. 2010) have been shown to be ectomycorrhizal with Himalayan blue pine (*Pinus wallichiana*) from the northwestern Himalayan region of India.

2.3 Distribution and habitat

Most of the *Suillus* species known so far are documented from northern hemisphere, but some have been also reported from southern hemisphere associated with the introduced pine species (McNabb 1968; Watling and Gregory 1989; Dunstan et al. 1998). In Western Australia, *S. granulatus* and *S. luteus* are among the most abundant and frequently encountered *Suillus* species. In general *Suillus* species are confined to the temperate, boreal and Mediterranean regions, although there are few reports of their occurrence from tropical areas (Natarajan and Raman 1983; Halling and Mueller 2002). *Suillus* species are common root symbionts of members of the family *Pinaceae* and also some deciduous species (Singer 1986; Kretzer et al. 1996; Wu et al. 2000). Researchers have reported strong host specificity in basidiomycetes (Begerow et al. 2004; Shefferson et al. 2007) displaying different levels of specialization. Majority of the ectomycorrhizal (ECM) fungi are associated with a broad range of hosts (Trappe 1962; Molina et al. 1992; Kårén et al. 1997; Smith and Read 1997; Bruns et al. 1998; Horton and Bruns 1998; Cairney and Chambers 1999; Cullings et al. 2000; Kennedy et al. 2003), whereas others are associated with narrow range of hosts (Massicotte et al. 1994;

Molina and Trappe 1994; Kretzer et al. 1996). *Suillus* species exhibit narrow host range, which are almost exclusively associated with *Pinaceae* (Kretzer et al. 1996; Wu et al. 2000). Based on the ITS sequence analysis, Kretzer et al. (1996) have proposed that *Larix* association in the genus *Suillus* seems to be primitive and associations with pines, Douglas-fir, and hardwoods seem to be derived. In fact, host shifts of basidiomycetes are considered to be major driving forces in their evolution (Refrégier et al. 2008; Li et al. 2009; Vercken et al. 2010; Li et al. 2011; Rochet et al. 2011).

Both the habitats and the symbiotic hosts are crucial while considering the conservation of ECM fungi. Many *Suillus* species has entered regional red-lists due to habitat loss, loss of symbiotic hosts, climatic changes, pollution, catastrophes etc. A few *Suillus* species have been enlisted as endangered or vulnerable species under regional red lists (Arnolds and Kuyper 1996). *Suillus sibiricus* has been listed as threatened species by seven European countries (Dahlberg and Croneborg 2006). In addition, *S. flavidus, S. tridentinus*, *S. collinitus*, *S. plorans* and *S. lakei* are included in individual country's red-lists, as threatened species.

2.4 Abundance and persistence

Suillus species form abundant basidiocarps in nature and are easy to culture as compared to the other ECM fungi. Advantage of their abundance and easy culturing has been employed in several studies focusing on the genetic structure and genet sizes of *Suillus* populations (Fries 1987; Dahlberg and Stenlid 1990, 1994; Dahlberg 1997; Bonello et al. 1998; Zhou et al. 1999). Dahlberg and Stenlid (1990, 1994) studied the spatial distribution of *Suillus bovinus* clones isolated from basidiocarps collected in different aged *Pinus sylvestris* stands and found that the size of genets increased with increasing forest age, suggesting that this species spread its population by mycelial growth. Increase in size of genets and their persistence over many years have been also reported in *S. granulatus* (Jacobson et al. 1993), *S. variegatus* (Dahlberg 1997) and *S. pungens* (Bonello et al. 1998) populations. On the contrary, a few *Suillus* species with small genet sizes in young forests or after disturbance act as early colonizers and are suggested reproducing mainly by spore dispersal (Dahlberg and Stenlid 1995; Zhou et al. 1999; Bruns et al. 2002). Thus, *Suillus* species are good competitors that can persist for long time and extend their distribution either by mycelial growth or by spore propagation. These two ecologically favorable attributes, abundance and persistence, encourages *Suillus* species as potential candidates for mycorrhizal applications in forestry purposes (El Karkouri et al. 2006).

2.5 Taxonomy

The classical approach of basidiomycetes systematics mainly relies upon phenotypic examination of sporocarps supplemented by microscopy and *in vitro* culturing. In addition to the morpho-anatomical descriptions of sporocarps, morphology of spindle pole bodies and septa as well as the physiological and biochemical characteristics of basidiomycetes fungi have remarkably contributed towards the basidiomycetes systematics (Yang 2011). Morphological, biochemical and physiological studies some time led to many ambiguities during identification and classification of basidiomycetes due to the insufficiency of characters (Hibbett 2007). Therefore, an authentic, reliable and supporting taxonomic tool is crucial for the documentation and classification of the fungal biodiversity. The limitation of identification of basidiomycetes based on a few morphological and physiological characters can be overcome by the molecular methods, which are becoming increasingly important for studying taxonomic and phylogenetic relationships among the fungi. During last two decades, the emergence of various molecular and phylogenetic methods has greatly accelerated the study of molecular systematic of fungi. Especially, the invention of polymerase chain reaction (PCR) and the availability of large number of universal oligonucleotide primers specific to the fungi (Vilgalys and Hester 1990; White et al. 1990; Gardes et al. 1991; Gardes and Bruns 1993) have greatly increased our potential to study the fungal systematics (White et al. 1990; Bruns et al. 1992; Nei and Kumar 2000). Further, the advancements in statistical methods and computational technology have made the assessment of phylogenetic relationship easier and more convenient. Molecular techniques such as, DNA sequence analysis, RAPD, RFLP, AFLP etc., have provided valuable information for understanding relationship among different groups of basidiomycetes/fungi and their classification (Hibbett and Vilgalys 1993; Bunyard et al. 1994; Bresinsky et al. 1999; Drehmel et al. 1999; Liu et al. 1999; Zhang et al. 2004; Binder and Hibbett 2006; Buyck et al. 2008). Sequences derived from ribosomal DNA (i.e. nSSU and nLSU rDNA), mitochondrial DNA (mtDNA) and protein coding genes (e.g. tef1, rpb1, rpb2, gpd) have been used to investigate the diversity and molecular evolution of the fungi in number of studies (Swann and Taylor 1995; Fell et al. 2000; Lacourt et al. 20001; Lutzoni et al. 2004; Nuytinck et al. 2006, 2007; Matheny et al. 2007a, 2007b; Nuytinck and Verbeken 2007; Geml et al. 2009; Stubbe et al. 2010; Van de Putte et al. 2010; Park et al. 2013, 2014). Thus, phylogenetic analysis of DNA or protein sequences has evolved as a powerful tool in fungal systematics very quickly in the last 20 years.

Despite the fact that molecular methods have become prevalent and necessary in fungal taxonomy and systematics (Seifert 2009; Begerow et al. 2010; Nilsson et al. 2011), classical methods still are of equal importance in understanding the evolution of the basidiomycetes. In the 20th century, many hypotheses were postulated regarding the evolution of basidiomycetes based on morphology, ultrastructure, and structure of pigments or metabolites that have been shown to be true by molecular analysis in the last two decades (Yang 2011). Therefore, multiple methods, including classical as well as molecular, should be used to document and classify the diversity of basidimomycetes. Although there are number of molecular methods that can be used along with morphological method, but ribosomal DNA (rDNA) sequence analysis always have been a most widely used methodology for authentic and reliable identification of fungal species and have greatly enhanced our present knowledge on phylogeny and evolution of fungi. In the present work, a combination of classical and rDNA based molecular taxonomy is used to study the intra-genus variation within *Suillus* species that are representatives of conifer forests in north western Himalayan region of India and a phylogenetic analysis is performed to determine their evolutionary relationships.

2.6 Internal transcribed spacers (ITS) and its limitations

In eukaryotes, ribosomal DNA (rDNA) comprises of various coding genes (18S, 5.8S, 28S and 5S genes) that are clustered in tandem repeat units separated by spacers, *viz*. internal transcribed spacer (ITS) and intergenic spacer (IGS) regions (Fig. 2.1). The ribosomal coding genes and the spacers have evolved at different pace during the evolution and have been used as the key targets for fungal identification purposes. These regions own the benefits of high copy number, high magnitude of conserved coding regions and variable spacer regions. The coding regions of rDNA exhibit a higher magnitude of conserved region that confers greater homogeneity within the species than between the species (Arnheim 1983) and provide us with the sites for primer designing. For example, PCR primers for identification and detection of *Rhizopycnis vagum* were developed by Ghignone et al. (2003) based on the alignment of ITS sequences. On the contrary, the spacer regions show remarkable sequence variations even within the species. The variations in the spacer regions have provided framework for identification, differentiation and classification of different ranks of the global biodiversity of kingdom fungi. At the same time, differences at inter-specific or intra-specific level have proven fruitful in distinguishing difficult-to-identify taxa. Thus, the rDNA is a convenient region to design nucleic acid markers for taxonomic purposes. The most widely sequenced rDNA locus for molecular systematics of fungi at the subgeneric level for species identification is the ITS region (Horton and Bruns 2001; Bridge et al. 2005). The ITS region comprises of the 3’ end of the 18S gene, the ITS1 spacer, the 5.8S gene, the ITS2 spacer and the 5’ end of the 28S gene (Fig. 2.1). In recent years, it has been consistently used for molecular systematics, phylogeny and ecology of fungi at the species level as well as within the species.

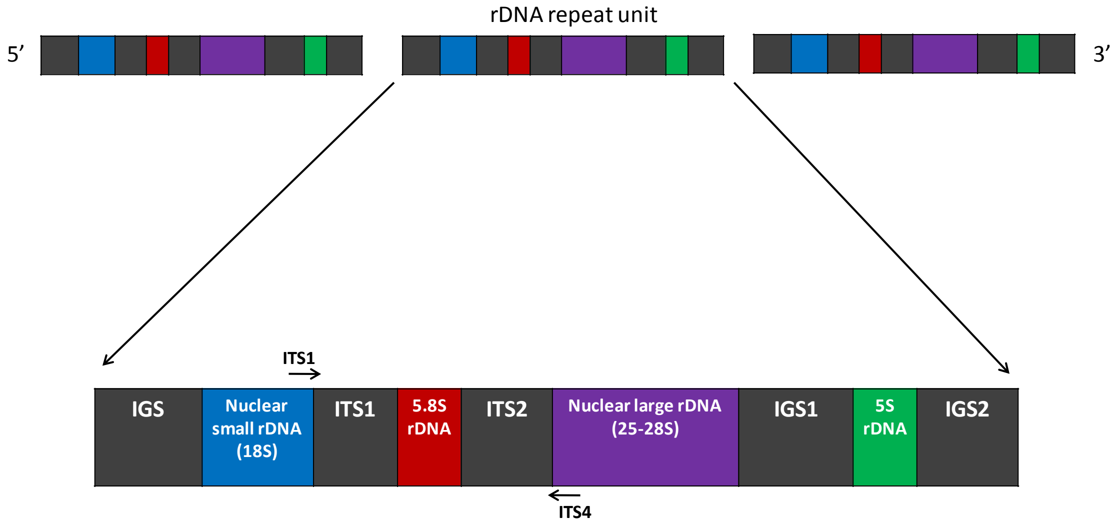


Fig. 2.1 Diagrammatic representation of fungal ribosomal DNA (rDNA) repeat unit. The universal primers used to amplify the internal transcribed spacer (ITS) region in this study are represented by arrows in 5’ to 3’ direction

Sequence analysis of ITS region in fungi, generally divulges a high level of inter-specific polymorphism and low or infrequent intra-specific divergence (Liu et al. 1997; Martin et al. 1998; Manian et al. 2001; Horton 2002; Singla et al. 2004; Leonardi et al. 2005; Mello et al. 2005; Park et al. 2014). For example, Manian et al. (2001) examined genetic diversity of common European *Suillus* species based on ITS data and found that *S. plorans* and *S. bovinus* exhibited maximum inter-specific divergence (13.6 and 13.3, respectively) when compared to other *Suillus* species. Analogously, the inter-specific divergence between a few pairs of *Suillus* species was found low, e.g. 2.4 between *S. collinitus* and *S. granulatus* and 3.8–4.2 between *S. plorans* and *S. variegatus*. Intra-specific divergence in ITS region differs considerably among the fungi, which ranges from 0-2% in some fungi (Gardes et al. 1991; Cooke et al. 1999; Manian et al. 2001) to 15% in others (Muthumeenakshi et al. 1998; Roy et al. 1998; Balardin et al. 1999; Manian et al. 2001). Phylogenetic analysis of *Suillus* species by Manian et al. (2001) revealed that *S. laricinus* (0.5–4.4) and *S. granulatus* (0–11) from Europe, Asia and North America showed high degrees of intra-specific divergence. On the other hand, *S. bovinus*, *S. variegatus* and *S. luteus* showed very low intra-specific divergence (0–0.7). Similarly, intra-specific homology and species-specific conservation of ITS region has been illustrated in different isolates of some *Suillus* species, such as *S. collinitus, S. mediterraneensis, S. luteus, S. albidipes*, *S. brevipes, S. tomentosus,* and *S. umbonatus* (Horton 2002; Beatriz et al. 2006).

Discrepancies between morphological and molecular identification of many ECM fungi including *Suillus* species have been revealed by many authors (Kretzer et al. 1996; Kårén et al. 1997; Farmer and Sylvia 1998; Wipf et al. 1999; Manian et al. 2001; Bruns et al. 2010). A review on exploration of physiology and biodiversity of ECM fungi was made by Buscot et al. (2000) who hypothesized four possible mechanisms for contradictions between phenotypic and genetic characters, which include paucity of phenotypic characters for species delimitation, intra-specific phenotypic flexibility, convergent evolution and cryptic speciation. The phylogenetic analysis based upon ITS data separated *S. granulatus* isolates from Europe, Asia, and North America into different groups (Kretzer et al. 1996; Manian et al. 2001; Bruns et al. 2010), probably suggesting that a cryptic speciation process has taken place between these isolates. It was suggested that the isolates reported as *S. granulatus* from Europe, Asia, and North America might be representing at least two different taxa. On the contrary, the ITS locus may be unsuccessful in distinguishing some well identified phenotypic species. Bruns et al. (2010) have mentioned that a few pairs of *Suillus* species (*S. pseudobrevipes* and *S. volcanalis*, *S. glandulosipes* and *S. neoalbidipes*, *S. brevipes* and *S. weaverae*) are not distinguished by the ITS locus. This might be due to over-description (=synonymy) or to the lack of ITS divergence among the sibling species. Thus, there are limitations of using ITS locus for species-level determinations in the genus *Suillus*.

Irrespective of the ITS limitations mentioned above, we have selected the ITS region for molecular identification and phylogenetic examination of the *Suillus* species, mainly for two reasons. First, the ITS region has been sequenced from large number of *Suillus* species from different regions of the world (Kretzer et al. 1996; Kretzer and Bruns 1997; Wu et al. 2000; Manian et al. 2001; Beatriz et al. 2006; Feng et al. 2008; Bruns et al. 2010; Sarwar et al. 2011, 2012; Sarwar and Khalid 2014). Second, several investigations on phylogenetics and bio-geographical analysis of the genus *Suillus* have demonstrated the advantage of using nuclear ribosomal RNA (nrRNA) region to diagnose and figure out the phylogenetic relationships among the species in this genus

(Kretzer et al. 1996; Wu et al. 2000; Manian et al. 2001; Feng et al. 2008). Until now, there was no study documenting the ITS sequences of *Suillus* species from India and this study will provided a phylogenetic framework for biogeographic analysis of Indian *Suillus* species as well as to infer phylogenetic relationships at the species level in this genus. Further, the ITS data may be a successful tool for distinguishing the cryptic species within this genus.

2.7 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) is a method for differentiation of organisms in which restriction patterns are obtained by the cleavage of DNA with restriction enzymes, followed by the segregation of the resulting fragments by gel electrophoresis to produce RFLP patterns. The technique is often applied to the fields of molecular ecology and systematics of fungi for species delimitations. Molecular ecological studies on ECM fungi have mainly employed restriction analysis of the ITS region (Horton and Bruns 2001) for identification and differentiation at the species level. Comparisons of ITS-RFLP patterns is an extremely simple, subtle and widely used method for determining inter-specific similarities or dissimilarities. Usually, two or three restriction enzyme patterns are adequate to discriminate most of the species (Gardes and Bruns 1996; Dahlberg et al. 1997; Pritsch et al. 1997, 2000; Mahmood et al. 1999; Methvyn et al. 2000).

Diversity of ECM symbionts associated with *Pinus halepensis* trees after out-planting at a fire-disturbed site (El karkouri et al. 2004) and containerized *Pinus nigra* trees established naturally (El karkouri et al. 2005) have been investigated in Mediterranean region of France. The nuclear ribosomal ITS region was PCR amplified with ITS1 and ITS4 primers and amplified PCR products were cut at least with three restriction enzymes, such as *Hinf*I, *Msp*I, *Mbo*I, and *Taq*I. Molecular typing using PCR-RFLP analysis and sequencing of ITS region of the nuclear rDNA detected twelve ITS-RFLP taxa on non-inoculated, *S. collinitus*-inoculated, and naturally regenerated trees in a fire-disturbed *P. halepensis* plantation (El karkouri et al. 2004). The ECM fungus *S. collinitus* was found to be the most dominant (45.8–59.7%) symbiont in all three treatments indicating its strong ectomycorrhizal competitivity in *P. halepensis* plantation of Mediterranean region. *Suillus mediterraneensis* occurred at moderate frequency (11.7–31.9%) and was confined to non-inoculated and naturally regenerated trees, suggesting its low ectomycorrhizal competitivity in contrast to *S. collinitus*. Dominance of both these *Suillus* species in *P. halepensis* plantation supports them as prospective ECM fungi for Mediterranean *P. halepensis* forest management. Ten other ITS-RFLP taxa observed on *P. halepensis* trees were rare (0.0–9.6%), demonstrating their poor competitivity against both the *Suillus* species. In naturally established *P. nigra* nursery, RFLP patterns of ITS region resulted in typing of four ITS-RFLP taxa (El karkouri et al. 2005). All of them were identified as *Boletales*, namely *Rhizopogon rubescens*, *R. luteolus*, *S. bovinus* and *S. variegatus*. *Rhizopogon rubescens* was most abundant (37.5%) among all the ectomycorrhizal *Boletales* detected, while *S. bovinus* (25%) and *S. variegatus* (26.4%) appeared at moderate frequency. The ECM symbiont *R. luteolus* was rare and occurred with low frequency (2.8%) on *P. nigra* seedlings. In addition, the ITS-RFLP along with inter simple sequence repeat (ISSR) amplification and specific sequence-characterized amplified region (SCAR) sequencing allowed the detection of *S. collinitus* strain Sc-32 on *P. halepensis* seedlings after inoculation and outplanting in a Mediterranean plantation (El karkouri et al. 2006). The survival of *S. collinitus* Sc-32 was monitored for 56 months after inoculation and it was demonstrated that inoculant can survive on seedlings even after 4 years of outplanting. This suggested that *S. collinitus* Sc-32 is a suitable and persistent inoculant for *P. halepensis* forestry in adverse Mediterranean ecosystems.

Beatriz et al. (2006) studied the molecular characterization of nineteen *Suillus* isolates from different pine forests of central Spain. Variation within ITS region of *Suillus* was examined by PCR (using primers ITS5 and ITS4B) coupled with RFLP analysis. PCR products amplified from all the *Suillus* isolates were approximately 800 bp in length. Restriction digests were produced using three different restriction enzymes *i.e.*, *Alu*I, *Hinf*I and *Tqq*I. Cluster analysis based on restriction digests grouped the isolates into six different groups. Different isolates of the same species were grouped together, confirming the low intra-specific variability in the ITS region. Moreover, the sequence analysis of ITS region resulted in accurate identification of some *Suillus* isolates, which were misidentified earlier by morphological methods. These studies discussed herein confirm the potential of ITS-RFLP for the identification, molecular characterization, species delimitation and ecological studies of *Suillus* species. Using this technique for documentation of *Suillus* taxa from an un-explored region, like northwestern region of India, will further extend our knowledge on taxonomic diversity of the genus *Suillus*.

2.8 Ectomycorrhizae of *Suillus* species

According to an information system for Characterization and **DE**termination of **E**cto**MY**corrhizae (DEEMY) (2004–2014), ectomycorrhizae of eight *Suillus* species have been characterized from different parts of the world. Among them, ectomycorrhizae for five *Suillus* species (*S. bovinus*, *S. collinitus*, *S. plorans*, *S. sibiricus* and *S. variegatus*) have been described in association with *Pinus* species (Uhl 1988; Agerer 1990; Treu 1990; Mleczko and Ronikier 2007) and rest three have been characterized from *Larix* roots. Recently, ectomycorrhizae of *S. flavidus* with *P. wallichiana* were studied from Pakistan (Sarwar et al. 2012). In context of India, ectomycorrhizae formed by different ECM fungi, including *S. brevipes*, on *Pinus patula* roots have been described from southern region of India (Mohan et al. 1993a, 1993b, 1993c). All these studies reveal that ectomycorrhizal systems of different *Suillus* species vary remarkably in morphology and anatomy. Mleczko and Ronikier (2007) have demonstrated that the ectomycorrhizal features of *Suillus* species are more congruent with their phylogenetic relationships as compared to the classical basidiocarp-based systematics.

2.9 Role of ectomycorrhizal fungi and *Suillus* species in pine establishment

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 (Garbaye 2000; Chalot et al. 2002; Hall 2002). There are many studies demonstrating the positive effects of ECM fungi on growth and nutrient contents of plants, especially the pine trees. Following mycorrhizal inoculation of *P. sylvestris* with *Boletus* species, a significant increase in dry weight and phosphorus content of mycorrhizal seedlings was observed by Mejstřík (1975). Inoculation of *Pinus rigida* seedlings with *Pisolithus tinctorius* showed superior growth and normal foliar ion composition even under phosphorus limitation compared to the un-inoculated seedlings (Cumming 1993). *Pinus densiflora* seedlings inoculated with *Tricholoma matsutake* showed a significant increase in total seedling dry weight compared to the control treatments (Guerin-Laguette et al. 2004). In a nursery experiment, Rincón et al. (2005a) found *Rhizopogon roseolus* as the best ECM fungi among the five ECM fungi tested for production of containerized mycorrhizal *Pinus pinea* seedlings. Compared with non-mycorrhizal seedlings, *P. densiflora* seedlings inoculated with different ECM fungi in single or multiple treatments exhibited increased seedlings growth (Sim and Eom 2006; Dalong et al. 2011) and nutrient contents (Dalong et al. 2011). Similarly, ectomycorrhizal inoculation of *P. wallichiana* seedlings with different ECM fungi showed an increase in growth, biomass and nutrient contents of the mycorrhizal seedlings (Dar et al. 2007, 2010; Ahangar et al. 2012; Itoo and Reshi 2014a). Additionally, highest plant growth response of pine seedlings has been observed when treated with a combination of multiple ECM fungi in contrast to single ECM fungus (Sim and Eom 2006; Dalong et al. 2011; Ahangar et al. 2012; Sousa et al. 2012).

While considering the ectomycorrhizal *Suillus* species, the fungi have been shown to promote plant height, root biomass, shoot biomass as well as nitrogen and phosphorus uptake in pine trees (Read and Boyd 1986; Bending and Read 1995; Colpaert et al. 1999; Beatriz et al. 2006; El Karkouri et al. 2006). Timonen et al. (1997) investigated root colonization and growth responses of *P. sylvestris* seedlings after inoculations with indigenous genets of *S. bovinus* (SBK4b and SBK5b) and *S. variegatus* (SVK3b), both in single inoculated treatments (SITs) and multiple inoculated treatments (MITs). All the mycorrhizal seedlings in SITs and MITs exhibited significant positive plant growth responses as compared to un-inoculated seedlings. Short-term phosphorus uptake rates in ectomycorrhizal and non-mycorrhizal roots of *P. sylvestris* seedlings have been studied following interactions with four ECM fungi, namely *Paxillus involutus*, *S. luteus*, *S. bovinus* and *Thelephora terrestris* (Colpaert et al. 1999). The results revealed a marked increase in phosphorus-uptake capacity of all the mycorrhizal plant root systems with heterogeneity in affinity for phosphorus-uptake among the different ECM fungi. Increased phosphorus uptake has also been shown in mycorrhizal *Pinus radiata* seedlings infected with *R. rubescens* and *S. luteus* (Liu et al. 2008). *Suillus collinitus*, forming ectomycorrhizas mainly with *P. halepensis* trees (Beatriz et al. 2006; El Karkouri et al. 2004) in harsh Mediterranean environments with water and nutrient poor soils, is used as a persistent mycorrhizal inoculant in nurseries and experimental plantations to improve pine growth, mineral nutrition and survival (El Karkouri et al. 2006). Moreover, stimulation of bacterial survival in *S. granulatus* colonized root elongation zone and their synergistic positive effect on *P. halepensis* seedlings has been observed by Rincón et al. (2005b). Furthermore, *Suillus* isolates exhibit metal tolerance to many toxic metals, such as Zn, Cu and Cd (Colpaert et al. 2000, 2004; Adriaensen et al. 2005) and these metals tolerant *Suillus* isolates have been shown to protect mycorrhizal pine seedlings from metal stress (Colpaert and Van Assche 1993; Adriaensen et al. 2004, 2005; Krznaric et al. 2009). Thus, metal adapted *Suillus* isolates can also be used as an excellent mean for large-scale afforestation and regeneration of pine seedlings at metal polluted and industrial sites.

2.10 Selection of efficient ectomycorrhizal isolates

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 (Dahlberg and Finlay 1999; Cazzoli 2002, Beatriz et al. 2006). 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 (*P. halepensis*) with an aim to select suitable isolates for afforestation programmes (Beatriz et al. 2006). Although there are a few studies reporting positive effects of different ECM fungi on growth, biomass, and nutrient contents of *P. wallichiana* seedlings (Dar et al. 2007, 2010; Ahangar et al. 2012; Itoo and Reshi 2014a), 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, it is of utmost importance to isolate local *Suillus* species and study their effects on plant growth so as to select suitable isolates for mass inoculum production.

2.11 *Pinus wallichiana* and need for indigenous ectomycorrhizal inoculants

Pinus wallichiana A.B. Jacks., commonly known as blue pine, is a five needle pine with an average height and width ranging from 30–36 m and 2.5–3.0 m, respectively (Troup 1921). Blue pine is native to the Himalayan region and occurs naturally in moist and dry temperate forest ecosystems of many adjacent countries of the region, such as Afghanistan, Pakistan, India, Nepal, Bhutan, Myanmar and China (Troup 1921; Critchfield and Little 1966). In these diverse forest ecosystems, it is distributed from middle to high altitudinal zones ranging from 1500–3600 m and extends longitudinally from 69^{o}–75^{o}E and latitudinally from 26^{o}–36^{o}N (Thapliyal et al. 2008; Singh and Thapliyal 2012). *Pinus wallichiana* is one among the most important and over-exploited pine species in the Himalayan region, which is mainly used as a source of commercially valuable timber in variety of purposes, such as construction, agriculture, horticulture, furniture manufacturing, fuel wood, religious practices etc. In terms of timber usage, blue pine is considered next to deodar (*Cedrus deodara*) among the coniferous plants of

Himalayas. Additionally, the species is important as it is also known for its turpentine and resin yield (Coppen et al. 1988; Shuaib et al. 2013). Thus, the blue pine holds a great potential for socio-economic uplift of people inhabiting the Himalayan regions. Recently, *P. wallichiana* has attained international attention for its resistance to blister rust disease (Khan 2004; Lu et al. 2007; King et al. 2010). Pertaining to its economic, aesthetic and ecological importance, *P. wallichiana* is a promising pine species for forestry programmes.

Pine trees are mycotrophic and mainly rely on mycorrhizal fungi for their early establishment, survival and growth (Smith and Read 1997). There is a relatively extensive literature (Cumming 1993; Timonen et al. 1997; Rincón et al. 2005b; Sim and Eom 2006; Dar et al. 2007; Ahangar et al. 2012; Sousa et al. 2012) reporting positive effect of ECM fungi on pine seedlings growth and survival under nursery conditions. These studies reveal that root colonizing and plant benefitting abilities of different ECM fungal strains are variable and it is advantageous to select specific strains as inoculants for production of mycorrhizal seedlings in nurseries. Selection of suitable ECM fungal isolates for afforestation practices in a defined area is a highly resource-consuming task. Moreover, the competition between different ECM fungi plays a significant role in the structuring of ectomycorrhizal assemblages (Koide et al. 2005). The survival of introduced inoculants in the nurseries or forest may get challenged by other indigenous ECM fungi, which further present a crucial problem in selection of mycorrhizal inoculants (Browning and Whitney 1992; Le Tacon et al. 1992, 1997; El Karkouri et al. 2002; Quoreshi et al. 2008). A decrease in inoculated ECM fungi after two years of inoculation has been observed in black spruce and jack pine seedlings (Browning and Whitney 1992). Similarly after five years of outplanting, most of the ECM fungi inoculated to conifer and hardwood species were replaced by several indigenous ECM fungi (Quoreshi et al. 2008). On the contrary, the efficiency of eco-specific and species-specific native ECM isolates is always superior than the introduced ones due to their environmental adaptation to the concerned site. The local isolate *S. collinitus* has been shown to survive on inoculated *P. halepensis* seedlings even after 56 months of inoculation (El Karkouri et al. 2006) and therefore used as a mycorrhizal inoculant in Mediterranean plantation. Improvement of *P. halepensis* seedlings establishment with selected *S. collinitus* isolates as inoculants has been also observed by Rincón et al. (2007) after 2 years of outplanting in a degraded gypsum soil. Kennedy et al. (2007) have suggested that the outcomes of ectomycorrhizal competition are strongly influenced by the local environment under which it occurs. Thus, ecological adaptability of introduced ECM fungi to the transplantation site is an important criterion to select well adapted ECM isolates for nursery inoculation programmes. For successful afforestation of a defined region, it is therefore advisable to identify and select native and efficient ECM strains for inoculum production, which are best adapted to the local environmental conditions. This practice, often known as "mycorrhization control", generally improves survival, field performance and plant productivity of mycorrhizal seedlings (Castellano and Molina 1989; Marx and Cordell 1989; Selosse et al. 2000; Rossi et al. 2007). In view of the above, the present investigation aimed at evaluating the effectiveness and influence of various indigenous *Suillus* isolates on growth, biomass and nutrient contents of *P. wallichiana* seedlings was undertaken.

2.12 Extracellular enzymes of ECM fungi involved in nitrogen and phosphorus uptake

Nitrogen and phosphorus are the major growth limiting nutrients in most of the natural forest soils. In these forest soils, nitrogen and phosphorus are present mainly as organic macromolecules that are not directly available to plants or fungi. Due to such nutrient constraints in many forest ecosystems, formation of mycorrhizas is considered a vital symbiotic interaction as they have been frequently reported to increase nutrients content in mycorrhizal plants as compared to non-mycorrhizal plants (Chalot et al. 2002; Smith and Read 2008). Mycorrhizal fungi allow access to unavailable forms of nutrients, especially phosphorus and nitrogen, by breaking down the organic macromolecules into simple accessible forms. ECM fungi utilize nutrients from organic compounds of soils by producing extracellular enzymes (Aučina et al. 2007; Courty et al. 2009, 2010b; Pritsch and Garbaye 2011). In light of the ecological importance of organic nutrient resources in many forest ecosystems and the potential of extracellular enzymes secreted by ECM fungi to break down these organic macromolecules into plant accessible forms, a few extracellular enzyme activities of *Suillus* isolates were evaluated in the present study so as to check their efficiency for pine establishment and afforestation.

Nitrogen uptake: Nitrogen (N) is a primary plant nutrient essential for plant growth and development. About 95% of soil total N is associated with the soil organic matter (proteinaceous material, amino acids, amino sugars, heterocyclic N compounds etc.) that is unavailable to plants. Rest of the soil N is present as nitrate (NO_3^-), nitrite (NO_2^-) and the ammonium (NH_4^+) ions, which constitute the major inorganic pool of soil total N (Schulten and Schnitzer 1998). Most of the plants utilize N in nitrate form and a few in ammonium (NH_4^+) form. The soil organic matter is continuously being converted to inorganic forms, by soil micro-flora, in a process termed as ‘mineralization’. The task is accomplished by a number of hydrolytic enzymes produced by the soil microbes. Most of the major hydrolytic enzymes involved in the mobilization of N from organic matter have been detected in ericoid and some in ECM fungi (Leake 1996; Chalot and Brun 1998). Among different hydrolytic enzymes, extracellular proteases are considered as key enzyme for mineralization of organic matter (Ramstedt and Söderhäll, 1983; Nygren et al. 2007) as 40% of the total soil N is present as protein N (Schulten and Schnitzer 1998). Numerous studies have highlighted the production and/or characterization of extracellular protease in ericoid and ECM fungi (Ramstedt and Söderhäll, 1983; El-Badaoui and Botton 1989; Leake and Read 1990a; Zhu et al. 1990, 1994; Leake and Read 1991). The efficiency of ericoid and ECM fungi to promote organic N uptake is correlated mainly with the extracellular protease activities (Bajwa et al. 1985; Leake and Read 1989; Leake and Read 1991; Leake 1996).

The ability of ECM fungi to secret hydrolytic enzymes, assimilate the products of hydrolytic degradation and mobilize the N from soils is considered possibly to have a great impact on the dynamics of organic N utilization in diverse forest ecological systems. The potential role of ECM fungi in assimilation and mobilization of N from organic matter has been illustrated by many authors (Abuzinadah et al. 1986; Abuzinadah and Read 1986; Bending and Read 1995; Martin and Lorillou 1997; Martin et al. 2001). The capability of several ECM fungi to utilize protein and mobilize its N to *Pinus contorta* plants has been also demonstrated (Abuzinadah et al. 1986; Abuzinadah and Read 1986). Moreover, the protease activities has been shown in many ECM species, such as *Amanita rubescens*, *Amanita muscaria*, *Cenococcum geophilum*, *Hebeloma* spp., *Lactarius subdulcis*, *P. involutus*, *S. bovinus*, *S. variegatus* etc. (El-

Badaoui and Botton 1989; Maijala et al. 1991; Leake 1996; Tibbett et al. 1999; Nehls et al. 2001). Recently, extracellular protease activity of 32 different species of ECM fungi was studied and 29 of the total species were found to exhibit extracellular protease activity (Nygren et al. 2007). Thus, the protease excretion is a prevalent physiological trait of ECM fungi and this ability is of substantial significance for N uptake in forest ecosystems.

Apart from proteins, chitin is another polymeric source of organic N particularly in the forest soils. The potential sources of chitin in soils are fungal cell walls and arthropod exoskeletons. Degradation of chitin and transfer of chitin derived N to the host plant by the ericoid mycorrhizal fungus *Hymenoscyphus ericae* has been demonstrated (Mitchell et al. 1992; Kerley and Read 1995). Also, the utilization of chitin as N source by ECM fungi is a well-known phenomenon (Leake and Read 1990b; Lindahl and Taylor 2004). The ability of ericoid and ECM fungi to promote uptake of chitin derived N may be correlated mainly with the chitinolytic activities of chitinase enzymes secreted by the mycorrhizal fungi (Mitchell et al. 1992; Lindahl and Taylor 2004).

Phosphorus uptake: Phosphorus (P) is one of the nutrients essential for plant growth and plays a censorious role in photosynthesis, energy metabolism, synthesis of nucleic acids and membranes, respiration, enzyme regulation and nitrogen fixation (Raghothama 1999; Vance et al. 2003). The only form of P in soils directly available to plants is inorganic orthophosphate (Pi). Beside this inorganic Pi, soils also contain phosphorus bound to carbon-containing compounds (organic P) that accounts for 20 to 80% of the total phosphorus in the soils (Richardson 1994). Acid phosphatases (ACPases) or phosphomonoesterases are a group of extracellular enzymes that mineralize this organic

P reservoirs of soils into a plant accessible inorganic Pi (Smith and Read 1997). Thus, ACPase improve the phosphate nutrition of plants and have been reported from a number of ECM fungi (Ho 1989; Antibus et al. 1992; McElhinney and Mitchell 1993; Periasamy and Raman 1995; Raman et al. 1998, 2002; Tibbett et al. 1998, Conn and Dighton 2000; Wannet et al. 2000; Jayakumar and Tan 2005; Quiquampoix and Mousain 2005; Alvarez et al. 2006; Courty et al. 2006; Baghel et al. 2009; Hrynkiewicz et al. 2009; Nygren and Rosling 2009; Louche et al. 2010; Bechem 2013). Quiquampoix and Mousain (2005) quantified the ACPase activities in ten isolates of ECM fungi (*Laccaria laccata*, *S. collinitus* (three isolates), *S. granulatus*, *S. luteus*, *H. cylindrosporum* (two isolates), *P. involutu*s and *R. rubescens*) grown in a low-phosphate medium. Among them, only six isolates showed extracellular ACPase activity (*S. collinitus* (one isolate), *S. granulatus*, *S. luteus*; *H. cylindrosporum* (two isolates) and *R. rubescens*). The high level of extracellular ACPase activities was detected in four different fractions separated from culture medium of *H. cylindrosporum* after growing in a pure culture under P-starved conditions and thus, suggested to produce several isoforms of ACPases (Louche et al. 2010). Increased level of extracellular ACPase activity in *Hebeloma* species under P-starved conditions has been also reported by other authors (Tibbett et al. 1998; Quiquampoix and Mousain 2005). ACPase activity has been also shown in some *Suillus* species, such as *S. bovinus*, *S. granulatus, S. collinitus* and *S. luteus* (Timonen and Sen 1998; Quiquampoix and Mousain 2005; Cullings et al. 2008; Dar et al. 2010). Moreover, uptake and distribution of P by mycelia of *S. variegatus* strains (Timonen et al. 1996; Wallander 2000) have been demonstrated.

Phytases are the next important extracellular enzymes involved in release of inorganic Pi from organic P. Phytic acid (inositol hexaphosphate) is a principal constituent of organic P found in the soils (Richardson 1994). These phytates are more stable to decomposition and accumulate in soils (Lim et al. 2007). Ability of ECM fungi to degrade phytates of organic P is attributed to their phytase activities, which allow access to this more recalcitrant organic phosphorus resource (Antibus et al. 1992). Phytase activity of ten ECM isolates has been determined (Quiquampoix and Mousain 2005), out of which only four isolates exhibited extracellular phytase activity that includes one isolate of *S. collinitus* and *S. granulatus* each and two isolates of *H. cylindrosporum*.

2.13 Biochar

2.13.1 Biochar: Definition and properties

Biochar is defined as biomass-derived black carbon produced by the pyrolysis of biomass feedstock in partial or total absence of oxygen. Biochar is a solid, carbonaceous material, which displays a high surface area (Bird et al. 2008) and less concentrations of oxygen and hydrogen level (Abdullah and Wu 2009) as compared to the feedstock. It is a byproduct of the pyrolysis process, which is chiefly composed of recalcitrant organic C and other plant mineral nutrients retained from the original biomass feedstock. Actual elemental composition of biochar depends both upon the source feedstock as well as the pyrolysis conditions (Novak et al. 2009). Carbon contents of biochar vary from 172 g/kg to 905 g/kg (McElligott et al. 2011). Additional elements present in the biochar includes nitrogen ranging from 1.8 g/kg to 56.4 g/kg, phosphorus ranging from 2.7 g/kg to 480 g/kg, potassium ranging from 1.0 g/kg to 58 g/kg and varying amounts of other elements, e.g. oxygen, hydrogen, sulphur, and heavy metals (Goldberg 1985; Lehmann et al. 2003a, 2003b; Lima and Marshall 2005; Preston and Schmidt 2006; Novak et al. 2009).

A variety of organic feedstocks and different process conditions may be used to produce biochars, which results in products with a diverse range of physico-chemical properties (Baldock and Smernik 2002; Nguyen et al. 2004; Guerrero et al. 2005). In fact, most of the physical and chemical characteristics of the biochar are sensitive functions of pyrolysis feedstock type and process conditions, which subsequently effects the soil properties when used as additive for soil management (Gaskin et al. 2008; Amonette and Joseph 2009; Downie et al. 2009; Novak et al. 2009). Feedstock is a key factor in determining the physicochemical properties of biochar. Gaskin et al. (2010) studied the effect of peanut hull and pine chip biochars on soil nutrients and found that peanut-shell biochar had higher nutrient concentrations than pine chip biochar and increased the pH and nutrients of soil. On the contrary, pine chip biochar decreased soil pH and had little or no effect on soil nutrients. The pore size of biochars, which is possibly important in governing water holding and adsorption capacity of soil, also varies for biochars derived from different feedstocks and exhibits the architecture of the source feedstock (Day et al. 2005; Ogawa et al. 2006; Yu et al. 2006). Novak et al. (2009) derived biochars from different feedstocks such as, peanut hulls, pecan shells, poultry litter and switch grass and observed that they differed entirely in their physical and elemental characteristics. Just as source feedstocks alters the properties of biochar, so too does the pyrolysis temperature. It has been observed that the process temperature is the most significant process parameter. Atkinson et al. (2010) and Joseph et al. (2010) have reviewed the effects of process temperature on biochar properties. The carbon content of biochar derived from different feedstocks (Novak et al. 2009) increased, while the yield of biochar decreased with an increase in the pyrolysis temperature. Thus, the carbon content of biochar is inversely related to the biochar yield, when pyrolysis temperature is increased. However, above a definite threshold temperature, biochar yield may continue to decrease with no more increase in the carbon content (Sohi et al. 2010). The surface area (Day et al. 2005) and ash content (Novak et al. 2009) of biochar also increases as the temperature is raised and this increase is different in different feedstock types. Moreover, the pyrolysis temperature also affects the pH of biochar, which is generally acidic for low temperatures derived biochar and alkaline for high temperatures derived biochar (Lehmann 2007, Novak et al. 2009).

2.13.2 Historic usage and significance of biochar

Much of our present knowledge regarding biochar has been enlightened from *terra preta de índio* (TP) soils of Amazon. The TP soils are the highly fertile Amazonian dark soils of the central Amazon as compared to the surrounding low fertile soils and are supposed to be created approximately 1000 ybp as illustrated by the Archaeological records and radiocarbon dating (Sohi et al. 2010). High char content of Amazonian dark soils accounts for their greater fertility and dark color (Glaser et al. 2001). The Amazonian dark soils were created by pre-Columbian Indians (Smith 1980; Woods and McCann 1999) and the extent of char content in these soils indicates that the char has been added to the soil purposely so as to increase the fertility of low fertile Amazonian soils. The other cause of increased char content is considered the biochar derived from incomplete combustion of biomass resulting from domestic fires and agricultural burning practices. Studies focusing on the development of TP soils in the Amazon have shown that these soils display enhanced levels of char content relative to the adjacent soils (Sombroek 1966; Glaser et al. 2001; Lehmann et al. 2002), which is resistant to decomposition and can remain in the soil for thousands of years (Agee 1996; Lehmann and Rondon 2006). TP soils have also been reported from Ecuador and Peru in western South America (Lehmann et al. 2003a, 2003b) and from the savannas of South Africa (Bird et al. 1999). There is relatively extensive literature (Young 1804; Von Liebig 1878; Morley 1927, 1929; Santiago and Santiago 1989; Lehmann and Joseph 2009) documenting the applications of char in traditional horticultural practices and soil improvement from various countries. Use of charcoal for non-fuel purposes like soil amendment in agriculture and forestry has also been a traditional practice in many Asian countries (Okimori et al. 2003). Thus, it may be suggested that the addition of biochar to the soils was probably a worldwide practice to enhance the soil fertility and the gross crop production.

Several recent investigations and reviews on TP soils have emphasized the importance of biochar as a soil additive in long term storage of carbon in soils as well as overall increase in the crop yields (Glaser et al. 2002; Glaser and Woods 2004; Day et al. 2005; Lehmann et al. 2006; Marris 2006; Glaser 2007). In contrast to surrounding soils, these TP soils possess higher amounts of organic matter contents and nutrients (such as nitrogen, phosphorus, potassium, and calcium) as well as the elevated levels of CEC and pH (Sombroek et al. 1993; Glaser et al. 2001; Lehmann et al. 2003a; Liang et al. 2006). The improved nutrient status and enhanced fertility of these TP soils have engendered significant increase in crop yields as compared to the adjacent low fertile soils (Lehmann et al. 2002; Liang et al. 2006; Solomon et al. 2007). These findings have inspired the use of biochar as a propitious soil additive in recent years. Despite global traditional use of biochar, present interest in biochar only began in last few decades. Presently, Japan is the largest commercial producer of charcoal for soil amendments

(Okimori et al. 2003). Biochar exploration significantly escalated in Japan during 1980s (Kishimoto and Sugiura 1980, 1985). As a result, the consumption of carbon in Japan increased from 38,800 t (metric ton) in 1985 to 192,000 t in 1999, out of which the maximum consumption (30.6%) was mainly as soil amendments for agricultural purposes (Okimori et al. 2003).

2.13.3 Biochar and carbon sequestration

The addition of biochar to the soil can contributing towards soil C sequestration (Lehmann 2007; Laird 2008) by restoring the reducing carbon repositories (Lehmann et al. 2006) and increasing the long term storage of carbon in soils (Kuzyakov et al. 2009; McHenry 2009). Biochar produced from organic feedstocks mainly consists of stable aromatic forms of organic carbon and the degree of aromaticity is a function of source feedstock and pyrolysis conditions. Aromaticity is an important governing factor of biochar potential for C sequestration, which stimulates the practice of biochar addition to soils to sequester C (Lehmann 2007). When used as a soil amendment, the aromatic organic carbon of biochar is resistant to decomposition (Amonette and Joseph 2009) and leads to an increase in the recalcitrant C fraction of the soil. Thus, the biochars especially with high aromaticity are resistant to microbial mineralization (Glaser et al. 2002; Lehmann 2007; Novak et al. 2009) and apparently suitable for long-term C sequestration. Increase in soil C fraction further improves the soil quality because soil C plays a crucial role in maintaining chemical, biological and physical processes in the soil (Thompson and Troeh 1978; Stevenson 1994).

2.13.4 Impacts of biochar on plant growth

Soils can be amended with biochar addition to improve plant biomass, crop productivity and soil quality (Blackwell et al. 2009). Reviews on previous studies showed a broad range of biochar application rates and variable plant responses to biochar additions (Glaser et al. 2001, 2002). Glaser et al. (2001) have illustrated that low biochar additions (0.5 t/ha) generally tended to have positive impacts on various crop species, whereas higher biochar rates inhibits crop biomass and/or productivity. In fact, there is relatively extensive literature available documenting the impacts of biochar on plant growth and crop produvtivity (Iswaran et al. 1980; Kishimoto and Sugiura 1985; Chidumayo 1994; Mikan and Abrams 1995; Wardle et al. 1998; Gaur and Adholeya 2000; Hoshi 2001; Lehmann et al. 2002; Chan et al. 2007; Van Zwieten et al. 2007; Kimetu et al. 2008). Majority of these studies (Iswaran et al. 1980; Chidumayo 1994; Wardle et al. 1998; Hoshi 2001; Lehmann et al. 2002; Chan et al. 2007; Van Zwieten et al. 2007; Kimetu et al. 2008) illustrated the beneficial effects of biochar on plant biomass and/or crop yield as a result of biochar additions. Despite this, biochar additions do not always positively effects the soil quality and negative impacts on plant growth have also been revealed due to biochar additions by few authors (Kishimoto and Sugiura 1985; Mikan and Abrams 1995; Gaur and Adholeya 2000).

Furthermore, increased plant growth, nutrient uptake and crop yield have been observed when biochar was added in combination with fertilizer to the soil (Glaser et al. 2002; Lehmann et al. 2002; Yamato et al. 2006; Chan et al. 2007, 2008; Gundale and DeLuca 2007; Steiner et al. 2007; Van Zwieten et al. 2007; Asai et al. 2009; Blackwell et al. 2009; Gaskin et al. 2010). However, a study from Australia on wheat crop didn’t show any significant yield response (Blackwell et al. 2007). Thus, fertilizer additions do not always alleviate the negative impact of fresh biochar additions (Asai et al. 2009). It has been considered that due to high C/N ratios (up to 400) of biochar, it has potential to immobilize plant accessible N causing N deficiency in plants (Lehmann et al. 2006; Chan and Xu 2009; Lehmann and Joseph 2009).

2.13.5 Impacts of biochar on soil properties

Biochar has been used as a soil amendment due to its potential soil conditioning properties and ability to make some desirable changes in the soil physicochemical characteristics. Numerous studies have shown that the addition of biochar to the soil may be advantageous as it can improve soil physical, chemical, and biological properties, increase recalcitrant soil organic carbon fraction and enhance plant growth (Glaser et al. 2002; Lehmann et al. 2002; Lehmann et al. 2005; Rondon et al. 2005; Lehmann and Rondon 2006; Fowles 2007; Rondon et al. 2007; Blackwell et al. 2009; Chan and Xu 2009; Downie et al. 2009). The collaborative abilities of biochar to increase soil pH (Tryon 1948; Mbagwu and Piccolo 1997; Hoshi 2001; Glaser et al. 2002; Matsubara et al. 2002; Yamato et al. 2006; Rondon et al. 2007; Van Zwieten et al. 2007), improve physical properties (Iswaran et al. 1980; Brady and Weil 2004; Chan et al. 2007; Downie et al. 2009), and retaining soil nutrients and reducing leaching losses (Hoshi 2001; Lehmann et al. 2002, 2003a; Lehmann 2007) are the possible contributing factors responsible for increase in plant productivity.

Fowles (2007) have discussed different attributes of the black carbon that may possibly contribute to increase soil fertility and improve its nutrient status. First, biochar enhances soil cation exchange capacity thus increasing the availability of nutrients for plants. Second, the soil nutrients can bind to the biochar that prevents the subsequent nutrient run-off from the soil and reduces leaching of nitrogen into the water table. Thus, biochar improves the filtration of percolating soil water (Lehmann and Joseph 2009). Third, the porous nature of biochars provides appropriate conditions for growth and multiplication of soil micro-biota. Soil microorganisms can proliferate within these pores and get protected from soil predators (Saito 1990; Pietikäinen et al. 2000; Ezawa et al. 2002; Samonin and Elikova 2004), such as arthropods, nematodes and protozoan. Thus, biochar acts as a refuge for any soil microorganism colonizing biochar. Further, the soluble organic substrates and nutrients bound to black carbon provide nutrition to the soil microorganisms (Hamer et al. 2004; Atkinson et al. 2010). As consequences, increases in microbial biomass and activities of biochar amended soils have been reported by many authors (Wardle et al. 1998; Pietikäinen et al. 2000; Chan et al. 2008; Steiner et al. 2008; Kolb et al. 2007; Steinbeiss et al. 2009). Soil microbiota is considered as architects of soils (Rajendhran and Gunasekaran 2008) and many ecosystem services, such as the decomposition of organic matter, cycling of nutrients, and suppression of soil-borne diseases and pests, are closely linked to microbial activities and their functional traits (Brussaard 1997). In addition, the potential benefits of incorporating biochar into soil include improvement of soil water retention capacity (Piccolo et al. 1996; Downie et al. 2009) and neutralization of acidic soil (Van Zweiten et al. 2007) by increasing the soil pH.

2.13.6 Impacts of biochar on mycorrhizal fungi

Biochar amendment can transform the total fungal abundance as well as the activity of mycorrhizal fungi in the soils that can in turn affect the mycorrhizal colonization or mycorrhizal efficiency and lead to the altered plant growth. Several investigations have reported both increased root colonization (Harvey et al. 1976; Saito 1990; Ishii and

Kodoya 1994; Mori and Marjenah 1994; Ezawa et al. 2002; Matsubara et al. 2002; Yamato et al. 2006; Rillig et al. 2010) and stimulated plant growth (Vaccari et al. 2011; Robertson et al. 2012) in response to biochar. This may be attributed to elevated nutrients availability (Ishii and Kadoya 1994; Garcia-Montiel et al. 2000; Lehmann et al. 2003a; Gundale and DeLuca 2006; DeLuca et al. 2006; Nigussie et al. 2012) or improved soil physicochemical properties (DeLuca et al. 2006; Warnock et al. 2007; Chan and Xu 2009) as a result of biochar supplementations. As majority of the literatures available so far illustrate that biochar additions positively affect both mycorrhizas and plant growth, it is noteworthy to discuss possible mechanisms underlying biochar functioning.

Warnock et al. (2007) have explained four possible mechanisms, which describe that how biochar can positively affect mycorrhizal abundance and subsequent plant development. (1) Addition of biochar to the soil induces altered levels of soil physicochemical properties and nutrient availability. Biochar additions increases/decreases soil pH, increases cation exchange capacity (CEC), water holding capacity (WHC), and decreases bulk density. Although the nutrients content in biochar is small, but the alteration of soil physicochemical properties due to biochar addition further led to an increase in soil nutrient availability. This increased level of nutrients can promote the growth of mycorrhizal fungi and the subsequent mycorrhizal colonization of plant roots. (2) Biochar is a potentially diverse niche for large number of soil microorganisms. Biochar facilitate the growth of biochar colonizing bacteria, including mycorrhization helper bacteria (MHBs) and phosphate solubilizing bacteria (PSBs), by providing them reduced carbon compounds, nutrients and protection. Enhanced abundance of MHBs and PSBs in biochar treated soils may further increase the growth of mycorrhizal hyphae and amount of fungal accessible mineral nutrients, respectively. This ultimately results in increased plant root colonization and plant nutrition uptake. (3) Biochar additions can affect plant–mycorrhizal fungi signaling dynamics by adsorption/desorption of both signaling compounds (carbon dioxide, flavanoids, sesquiterpenes and strigolactones) as well as inhibitory compounds (allelochemicals, other toxic molecules). Thus, biochar can serve as signal reservoirs or sink. When the signaling compounds are adsorbed permanently by biochar, fungal hyphal growth and subsequently the fungal abundance decreases. However if biochar temporarily removes the signal molecules, desorption may take place later on when soil water enters the biochar particles. As a result of desorption, biochar particles can serve as secondary sources of signal molecules and stimulate mycorrhizal colonization of plant roots. Attenuation of allelochemicals or other toxic molecules due to biochar adsorption is also advantageous to mycorrhizal fungi and promotes plant root colonization. In addition to direct effects of biochar on signaling compounds, biochar can indirectly increase stimulating signals and subsequent fungal abundance by altering soil pH. (4) Biochar provides protection to root colonizing fungi and bacteria from soil predators. Thus, biochar serves as refuge from grazing species, such as arthropods, nematodes and protozoan. Phenomenon is purely physical and depends upon pore size of biochar particles. Many of the pores are large enough to get colonized by fungal hyphae and bacteria, but exclude their larger predators. Protection within biochar particles may promote abundance of MHBs, PSBs and mycorrhizal fungi in soil, which further may led to an increase in plant root colonization. According to Warnock (2007), several of these mechanisms are hypothetical and it is difficult to mention which mechanism is likely to be most important under any given environmental conditions. At present, mechanism 1 is considered to most satisfactory due to vast literature available in its support (Tryon 1948; Ishii and Kadoya1994; Glaser et al. 2002; Lehmann et al. 2002, 2003a, 2005; Matsubara et al. 2002; Rondon et al. 2005; DeLuca et al. 2006; Gundale and DeLuca 2006; Lehmann and Rondon 2006; Yamato et al. 2006; Fowles 2007; Rondon et al. 2007; Blackwell et al. 2009; Chan and Xu 2009; Downie et al. 2009).

Although majority of literature shows that biochar applications can affect both mycorrhizas and plant growth positively, but this is not observed always. Warnock et al. (2010) have found that fungal abundance in roots of *Plantago lanceolata* either decreased or remained unchanged for all the five types of biochar amendments used. Moreover, Gaur and Adholeya (2000) found that the biochar supplementation limited the P uptake in *Zea mays* plants, thus negatively affecting the plant growth. Decrease in soybean yields due to biochar addition has been also reported by Kishimoto and Sugiura (1985). This reduction in root colonization and nutrients uptake because of biochar addition is possibly due to decreased nutrient availability or unfavorable nutrient ratios, such as, very high C/N ratio in the soils (Gaur and Adholeya 2000; Wallstedt et al. 2002). Considering these probabilities of negative effects of biochar amendments on mycorrhizal abundance and plant growth, substantial characterization and evaluation of biochar for plant growth needs to be done before it can be used for afforestation and land reclamation programmes. In the present work, synergistic influence of pine needle derived biochar amendments and *Suillus* mycorrhizal inoculations on *P. wallichiana* seedlings was studied as a practice for afforestation of forest soils.

Thus, the literature survey reveals that there are very few reports of the genus *Suillus* from India. Four species (*S. brevipes*, *S. pallidiceps*, *S. punctatipes* and *S.*

subluteus) have been documented from the southern part of India and only two from (*S. sibiricus* and *S. granulatus*) the northern region of India. Keeping in view the importance of molecular as well as classical methods, a combination of classical and rDNA based molecular taxonomy is used in the present work to study the intra-genus variations among the *Suillus* species that are representatives of conifer forests in the northwestern Himalayan region of India. Further, there is no deposition of the ITS sequences of *Suillus* species from India in any nucleotide database. This study will obtain the ITS sequences for the Indian *Suillus* species and provided a phylogenetic framework for biogeographic analysis of these species. Along with documenting the diversity, it is of great significance to conserve the biodiversity, which can be achieved by isolating and preserving the *Suillus* isolates. In context of India, there is no deposition of any *Suillus* isolates in any internationally recognized culture bank. The present study is focused on isolating the *Suillus* cultures with aim to evaluate their mycorrhizal capacity for improvement of *P. wallichiana* growth. The abilities of indigenous *Suillus* species (except for *S. granulatus*) and biochar additions to promote growth of *P. wallichiana* have not been evaluated, so far. In view of this, there is considerable work that remains to be done especially with reference to *Suillus* diversity, taxonomy, culture isolation and evaluation of their mycorrhizal capacities (with or without biochar addition), because of which the present work has been undertaken.

Chapter 3

Materials and methods

3.1 Geographical region surveyed

3.1.1 Northwestern Himalayan terrain of India

In the present study, different conifer forests of the northwestern Himalayan terrain (Fig 3.1) of India were surveyed in order to explore *Suillus* diversity of this region. The region is a part of the Himalaya that is recognized as one among the 34 global biodiversity hotspots in the world (Conservation International 2007), consequently reflecting its wide range ecological significance. It is situated between 28°43’–37°05’N latitude and 72°31’–81°03’E longitude covering a geographical area of 331, 392 sq km (India State of Forest Report 2011), which constitute about 10% of the country’s total geographical area. The area comprises of three states namely, Jammu and Kashmir (J.&K.), Himachal Pradesh (H.P.) and Uttarakhand (U.K.). Jammu and Kashmir is located between 32°17’–37°05’N latitude and 72°31’–80°20’E longitude, H.P. between 30°22’–33°12’N latitude and 75°45’–79°04’E longitude, and U.K. between 28°43’–31°28’N latitude and 77°34’–81°03’E longitude. For the collection of *Suillus* basidiocarps, frequent excursions were undertaken to various localities of the northwestern Himalayas during monsoon season (July–September) from 2009–2013. Temporary laboratory arrangements, required for taxonomic examination of the basidiocarps and isolation of cultures, were always established near to the collection sites. Although, attempts were made to explore or cover the maximum area of the region but main districts from where *Suillus* specimens were found, include Anantnag, Ganderbal, Kulgam and Udhampur in J.&K.; Chamba, Kangra, Kinnaur, Kullu, Mandi, Shimla and Sirmaur in

H. P. and Chamoli, Dehradun, Nainital, Tehri Garhwal and Uttarkashi in U.K. (Fig. 3.1). Efforts were made to explore the maximum area for the collection of *Suillus* specimens.

Fig. 3.1 Map of northwestern Himalayan terrain of India, showing different districts of three states (Jammu and Kashmir, Himachal Pradesh and Uttarakhand) from where *Suillus* specimens were collected

3.1.2 Climatic conditions of northwestern Himalayan terrain

The climate of northwestern Himalayas varies considerably due to altitudinal variations. Kashyap et al. (2014) have divided the northwestern Himalayan region broadly into four agro-climatic zones, namely low hill subtropical zone (<1000 m), mid hill sub-montane zone (1000–2000 m), high hill wet temperate zone (2000–3000 m) and high hill dry temperate zone (>3000 m). The variation in the altitude is a key factor in determining the temperature of different zones. The average summer (May–June) temperature is around 30ºC in the low hill subtropical zone and about 25ºC in the middle Himalayas, which further decrease to 15–18ºC in upper hills of the middle Himalayas. The temperature is least during winter season (December–February). During winter, many parts of northwestern Himalayas receive moderate to heavy snow fall. Therefore winters are very cold, especially in middle and higher Himalayas, experiencing temperatures even below freezing point. The region beyond 4,880 m is always covered with snow and below freezing point.

Precipitation in the northwestern Himalayas occurs due to western waves during the months from October to May and due to southwest monsoon during July to September. Depending upon the altitude, different parts of northwestern Himalayas receive different amounts of annual rainfall varying from 600–3200 mm. Seventy five percent of the total rainfall occurs in the month of July, August and September i.e. monsoon season. Precipitation due to the monsoon is highest in the eastern parts of the northwestern Himalayas, which receives bulk of the monsoon winds but it goes on decreasing as the winds move towards western side. The monsoon showers also decreases towards north (Rakhecha et al. 1983; Bhutiyani et al. 2010). Siwalik Hills and the Pir Panjal Ranges receive highest rainfall, which gradually decrease in Great Himalaya, Zanskar, Ladakh and Karakoram ranges. Most of the *Suillus* species reported in the present study were found during monsoon season from the mid hill sub-montane to high hill wet temperate zone (1000–3000) of the northwestern Himalayas.

3.2 Collection, identification and characterization of *Suillus* basidiocarps

3.2.1 Collection of *Suillus* basidiocarps

Standard methodology employed for the collection and preservation of basidiocarps followed Atri et al. (2005). *Suillus* basidiocarps were collected from different places of the northwestern Himalayas mostly during the rainy season (July–September) of 2009–2013. The different localities of J.&K., H.P. and U.K. were visited frequently, especially in the months of July and August. The basidiocarps were dug out carefully using a sharp knife, wrapped in the wax papers and placed in the collection baskets. Attempts were made to collect the basidiocarps of all the developmental stages to get a fair range of macroscopic features. Usually, data regarding the habit, habitat and ecological parameters of the specimens, such as altitude, soil type, forest type and probable association were recorded in the field. All the collections were brought carefully to the temporary laboratory set up near to the collection sites.

3.2.2 Spore print

The color of the basidiospores *en masse* is called spore print. The spore print of each specimen was taken on white spore print paper. For this purpose, a fresh mature basidiocarp was taken and a triangular piece of pileus was cut with surgical blade and placed over the paper with tubes facing downwards. A water soaked cotton plug was placed near the cap and covered with a petridish. Mostly, the spore prints were ready in 4–5 h. The color codes of Kornerup and Wanscher (1978) were used for recording the color of spore deposit. Finally, the spore prints obtained were preserved in standard wrappers after drying them for a few minutes.

3.2.3 Macro-chemical color reactions

Macro-chemical reactions were performed on pileal cuticle and pileal flesh of mature basidiocarps. Pieces of pileal cuticle and pileal flesh (2–4 mm in size) were kept on glass slides and treated with 2–3 drops of reagents. Color change if any, was noted after 2–5 min. The reagents used for macro-chemical reactions were 2.5% KOH (w/v), 10% $FeSO_4$ (fresh, w/v), 14% NH_4OH (w/v) and conc. HNO_3. Baroni (1978) have studied the chemical spot tests of 6 genera of *Boletaceae* and concluded that pink to reddish color reaction of pileal flesh with KOH and NH_4OH distinguishes *Suillus* species from other *Boletaceae*.

3.2.4 Macroscopic features

Standard methodology and terminology used for describing the basidiocarps followed Corner (1972). The basidiocarps were photographed at the collection sites using digital camera and various macroscopic characteristics were examined immediately or after bringing the specimens to the temporary laboratory. The macroscopic characteristics were noted down to the "Field Keys" specially designed for the purpose. The color codes used for describing basidiocarp parts are in accordance with Kornerup and Wanscher (1978). The following are the macroscopic features for different parts of basidiocarps that were studied for identification purpose.

a. **Pileus:** size, shape, color, color change on bruising, surface features, cuticle peeling, marginal characters, presence or absence of partial veil.

b. **Hymenophore:** tubes color, color change, length, attachment (adnate, subdecurrent to decurrent), arrangement, crowded or distant, consistency, pores size, shape, color etc.

c. Stipe: size, shape, color, color change on bruising, consistency, surface features, presence or absence of glandular dots, any distinguishing feature at the base.

d. Context: color, color change after cutting or bruising, thickness, texture, taste, smell.

e. Annulus: presence, absence, color, persistence and disappearance at maturity.

3.2.5 Preservation

After noting down the macroscopic characteristics including spore prints and chemical spot tests, the specimens were dried in specifically designed portable hot air driers at 45–55ºC. The dried specimens were wrapped in cellophane envelopes containing 1,4-p-dichlorobenzene and naphthalene balls as insect repellants. The packed specimens were marked systematically and assigned specific collection number (Table 4.1). All the specimens have been deposited in the Herbarium of the Botany Department (PUN), Punjabi University, Patiala, India (Table 4.1).

3.2.6 Microscopic features

Anatomical features were observed from dried material by reviving the free hand sections either in water or in 3% KOH (w/v). Measurements were made at 1000× magnification with a calibrated ocular micrometer on an Olympus light microscope (Olympus, Japan). For this purpose, the sections were stained in lactophenol cotton blue (see Appendix I). Basidia were measured from the hymenophore of mature basidiocarps. For basidiospores, spores were obtained from spore prints and mounted on a slide with few drops of Melzer’s reagent (see Appendix I). The spore measurements exclude the length of apiculus and the basidium length excludes the length of sterigmata. Quotient value (Q=L/W) was calculated considering the mean value of length and width of 20 basidiospores. Microscopic line drawings of microstructures were made from rehydrated material with the aid of a mirror type camera lucida. The following microscopic features were examined.

a. **Spores:** size, shape, coloration in KOH and Melzer's reagent and granulation.

b. **Basidia:** size, shape, contents, number of spores, coloration in KOH and Melzer's reagent, and sterigmata heights.

c. **Cystidia (Pleurocystidia, Cheilocystidia, Caulocystidia):** size, shape, contents, coloration in KOH and Melzer's reagent and any other significant feature.

d. **Clamp connections:** generally absent on the hyphae of *Suillus* basidiocarps.

e. **Trama:** generally divergent and gelatinous in the genus *Suillus*.

3.2.7 Isolation of pure cultures

Modified Melin-Norkrans (MMN) agar media (Marx 1969), Malt Extract (ME) agar (2% w/v) media and Potato dextrose agar (PDA) media (see Appendix I) 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.

3.2.8 Molecular studies

For molecular analysis of *Suillus* species, genomic DNA was extracted from dried basidiocarps and isolated cultures. The ITS region was amplified by PCR using the universal primers. PCR products were digested with specific restriction endonucleases to produce RFLP (Restriction fragment length polymorphisms) patterns. For sequencing purpose, PCR products were separated by electrophoresis on 1.5% agarose gels and purified using a Gel Extraction Kit (Qiagen, Germany). Purified products were sub-cloned and transformed into *Escherichia coli* DH5α cells and the clones containing the ITS insert were selected randomly and sequenced. The sequences obtained were analyzed using nucleotide BLAST, compared with the existing database and aligned with selected *Suillus* sequences using a multiple sequence alignment tool. Finally, a phylogenetic tree was constructed to study the interrelationships among the species.

3.2.8.1 DNA isolation protocol

Genomic DNA from dried basidiocarps and pure cultures was extracted according to Zhou et al. (1999), as follows:

1. Approximately, 100 mg of dried basidiocarp and/or culture mycelium was taken in a pre-chilled mortar and grinded using liquid nitrogen with the help of a pestle.
2. The powdered sample was immediately transferred to a pre-cooled Eppendorf tube (2.0 ml) followed by the addition of 1 ml of wash buffer (see Appendix I).
3. The mixture was vortexed briefly and resulting homogenate was centrifuged at 14000 rpm for 2 min. Washing and centrifugation of the recovered pellet was repeated at least five times in case of basidiocarps and two times in case of cultures.
4. For DNA extraction, 1 ml of 2% CTAB buffer (see Appendix I) was added to the washed pellet and incubated at 65°C for 1 h with intermittent (3–4 times) mixing of the suspension by inverting the Eppendorf’s tube gently.

5. To the resulting extract an equal volume of phenol: chloroform: isoamyl alcohol mixture (25:24:1, v/v) was added, mixed thoroughly for 15 min and centrifuged at 14,000 rpm for 15 min.
6. The upper aqueous phase was removed carefully without taking any interphase material and was further extracted twice with an equal volume of chloroform: isoamylalcohol mixture (24:1, v/v).
7. The final clear supernatant was transferred into a fresh tube and DNA was precipitated by adding an equal volume of isopropanol.
8. Precipitates were collected by centrifugation at 8,000 rpm for 5 min. Supernatant was discarded and DNA pellet obtained was re-dissolved in 100 µl of sterile Milli Q water.
9. For RNA removal, DNA solution was treated with 1 µl of 10 mg ml^{-1} ribonuclease solution (RNase, Fermentas) and incubated at room temperature for 30 min.
10. DNA was then precipitated by adding 60 µl of a PEG solution (see Appendix I) and harvested by centrifugation at 14000 rpm for 10 min.
11. DNA pellet obtained was washed with 70% ethanol and centrifuged at 8,000 rpm for 5 min.
12. Supernatant was removed and the pellet was dried for 20–30 min. Finally, the pellet was re-suspended in 50 µl of 1× TE buffer (see Appendix I) and stored at −20°C for further use.

3.2.8.2 Electrophoresis of DNA on agarose gels

Aliquots (5 µl) of isolated DNA were loaded on 0.7% (w/v) agarose gel made in 0.5× TBE buffer (pH 8.0, see Appendix I) using 6× gel loading dye (see Appendix I). To stain the DNA, ethidium bromide (0.5 µg/ml) was added to the agarose gel before pouring. The DNA was then electrophoresed at 40 V for 90 min and visualized on a UV transilluminator.

3.2.8.3 DNA quantification

The genomic DNA contents were quantified both by spectrophotometric as well as ethidium bromide fluorescent method.

a) Spectrophotometric quantification

The quality and concentration of extracted DNA in suspension was estimated by taking absorbance at A_{260} and A_{280} with an UV-vis spectrophotometer. DNA quality was evaluated by calculating A_{260}/A_{280} ratio. In general, for pure DNA the A_{260}/A_{280} ratio ranges from 1.8–2.0. Any deviation from this ratio indicates impurities in the isolated DNA. A_{260}/A_{280} ratios less than 1.8 indicate protein or phenol contamination and ratios greater than 2.0 indicate the presence of RNA in the extracted DNA. DNA concentration was calculated from A_{260}. Usually, a double-stranded DNA suspension giving an OD of 1.0 at a wavelength of 260 nm with a cuvette of 1 cm light path is considered equal to 50 µg/ml.

b) Ethidium bromide fluorescent quantification

DNA was analyzed electrophoretically in an agarose gel (0.7% w/v) prepared in 0.5× TBE, pH 8.0 and containing 0.5 µg/ml ethidium bromide. DNA was loaded using a 6× loading dye and then electrophoresed at 40 V for 90 min and visualized on a UV transilluminator. The quantity of DNA was estimated visually by comparing the intensity of DNA fluorescence with known DNA concentrations of lambda phage (Fermentas, USA) taken as references for DNA quantification.

3.2.8.4 PCR amplification of internal transcribe spacer (ITS) region

The internal transcribed spacer (ITS) region of nuclear ribosomal RNA (nrRNA) was amplified with the universal primers ITS1 (5’TCCGTAGGTGAACCTGCGG3’) and ITS4 (5’TCCTCCGCTTATTGATATGC3’) (White et al. 1990) using a PCR Thermal Cycler (Applied Biosystems, Singapore). PCR amplification was carried out in a volume of 50 µl containing 100 ng genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, and 2.5 units of *i*-taq^{TM} DNA polymerase (iNtRON Biotechnology, Korea). The thermal cycling conditions applied for the ITS region included an initial denaturation for 5 min at 95ºC followed by 34 cycles of 1 min at 94ºC, 1 min at 50ºC and 1.5 min at 72ºC and a final extension of 7 min at 72ºC. The reaction was stopped by cooling at 4°C for >5 min and reaction mixture was then stored at −20°C for further use. PCR products, 5 µl fractions, were mixed with 1 µl of 6× DNA loading Dye (Thermo Scientific, USA) and subjected to electrophoresis on 1.5% agarose gels. Bands were visualized on UV transilluminator and photographed with a gel documentation system (Quantum –ST4–3026/WL/26M, Vilber Lourmat, France).

3.2.8.5 RFLP analysis of ITS products

In order to obtain restriction patterns for different *Suillus* isolates, PCR amplified ITS products were subjected to RFLP analysis. Three different restriction enzymes, namely *AluI*, *HaeIII* and *MboI* (Fermentas, USA) were used in separate digestion reactions with the amplified ITS products.

1. An appropriate amount of DNA solution (0.5–1.0 µg) was taken in a sterile Eppendorf’s tube and final volume was made up to 17 µl with sterile MQ water.
2. 2 µl of corresponding 10× restriction enzyme assay buffer was added to the tube and mixed thoroughly.

3. 1 µl (2–5 units) of the restriction enzyme was added, mixed by tapping the tube.
4. The mixture was incubated at the recommended temperature for 2–3 h.
5. The reaction was stopped by adding 4–5 µl of 6× DNA loading Dye and mixed briefly by vortexing.
6. The digested DNA was electrophoresed through 2.0 % (w/v) agarose gels containing ethidium bromide for 4 h at 50 V. The restriction patterns were visualized and photographed using gel documentation system (Quantum–ST4–3026/WL/26M, Vilber Lourmat, France).

3.2.8.6 Purification and ligation of PCR products

For cloning purpose, amplified ITS products were purified and thereafter electrophoresed in 1.2% agarose gel. DNA bands were excised from the gel using sterile surgical blades and purified using a QIAquick gel extraction kit (Qiagen) as per the manufacturer’s instructions. Finally, the purified PCR products were collected in 30 µl of elution buffer, quantified as described in section 3.2.8.3 and stored at −20ºC for ligation purpose.

The purified ITS products were ligated into pTZ57R/T vector using InsTAclone, PCR cloning kit (Thermo Scientific, USA) as per the manufacturer’s directions (see Appendix I). The final reaction volume of 30 µl was incubated overnight (12–14 h) at 4°C. Five micro liters of ligation mixture was analyzed on 0.7% agarose gel to check out for successful ligation and rest was kept at −20°C for transformation purpose.

3.2.8.7 Genetic Transformation of ITS products into *E. coli* DH5α cells

a) Preparation of competent *E. coli* DH5α cells using $CaCl_2$

A freshly grown plate of *E. coli* DH5α was taken and a single colony was inoculated into 25 ml of Luria-Bertani (LB) broth medium (see Appendix I) in a 250 ml Erlenmeyer's flask. The culture was incubated overnight (14–18 h) at 37°C with vigorous shaking at 180 rpm. Then 250 μl of this overnight grown culture was transferred aseptically into 25 ml of fresh LB in a 250 ml Erlenmeyer's flask and incubated at 37°C with same shaking speed for 2–3 h. The OD at 590 nm was observed for the growing culture after every 1 h and culture was grown until OD_{590} approaches 0.5. Thereafter, the culture was transferred to sterile, 30 ml centrifuge tube and kept on ice for 10 min. The cells were collected by centrifugation at 8,000 rpm for 10 min at 4°C. The supernatant was removed and the pellet obtained was gently re-suspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice for 10–15 min. The cells were harvested again by centrifugation at 8,000 rpm for 10 min at 4°C, supernatant discarded, and pellet re-suspended in 1 ml of ice-cold 0.1 M $CaCl_2$. Calcium chloride treatment for 3–4 h induced a substantial transient state of "competence" in the *E. coli* cells. Five hundred micro liters of ice cold sterile 70% glycerol was added to the cells suspension, mixed gently while on ice, and 100 μl of competent cells were transferred to sterile, pre-chilled 1.5 ml Eppendorf's tubes and stored at −80°C for further use.

b) Transformation of competent cells by heat shock method

One hundred micro liters of competent cells stored in 1.5 ml Eppendorf's tubes at −80°C were taken out and kept on ice for thawing (10–15 min). The ligation mixture (3–5 μl) was added to each tube and mixed gently. After incubation on ice for 30 min, heat shock was given at 42°C in a water bath exactly for 2 min without shaking. The tubes were quickly kept back to an ice bath, chilled for 1–2 min and 900 μl of LB was added immediately to each tube. Tubes were incubated at 37°C for 45–60 min to allow the bacteria to express the antibiotic resistance marker encoded by the plasmid. Then 100 μl of transformed cells were spread on LB-ampicillin X-Gal/IPTG agar plates (see Appendix I) and incubated at 37°C for 12–16 h for selection of transformants.

3.2.8.8 Blue/white screening of transformed cells

X-Gal and IPTG were used to screen for colonies containing a recombinant plasmid. The multiple cloning sites (MCS) region of pTZ57R/T vector is present in the plasmid's lacZα gene. When the PCR product gets inserted into the cloning site, non-functional β-galactosidase is produced and the transformed bacterial colony is white. On the contrary, a functional β-galactosidase is produced if no insert is present and the transformed bacterial colony is blue. White colonies were picked using sterile toothpicks and short streaks were made on LB-ampicillin agar plates to save the clones for re-propagation. After streaking each toothpick was re-suspended in 18.25 µl of sterile MQ water taken in PCR tubes for 3–5 min and stored at 4°C for colony PCR.

3.2.8.9 Plasmid DNA isolation from recombinant bacteria by alkaline lysis method

The plasmid DNA from recombinant *E. coli* clones was isolated using alkaline lysis method. A single white colony was picked from a LB agar plate and inoculated into a loosely capped 30 ml test tube containing 2 ml of Luria broth supplemented with an appropriate amount of antibiotic (ampicillin, 50 μg/ml). The culture was incubated overnight (14–18 h) at 37°C with vigorous shaking at 180 rpm. Then, the culture was transferred to a 2.0 ml Eppendorf's tube and the cells were collected by centrifugation at

8,000 rpm for 5 min. Supernatant was discarded and the harvested cells were re-dissolved in 200 µl of ice-cold Solution I (see Appendix I). The mixture was vortexed vigorously to suspend the pellet completely into the solution. This was followed by addition of 200 µl of freshly prepared Solution II (see Appendix I) and the resulting suspension was mixed gently by inverting tube, four to five times. Vortexing was strictly avoided in this step. The tube was kept on ice for 5 min and then 300 µl of ice-cold Solution III (see Appendix I) was added. The tube was inverted gently to mix the contents and again kept on ice for 10 min. After centrifugation at 14,000 rpm for 10 min the upper aqueous phase was collected and extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v). The upper aqueous phase was taken in a separate tube and plasmid DNA was precipitated by adding 0.7 volumes of isopropanol, which was followed by centrifugation at 12,000 rpm for 10 min. The pellet obtained was suspended in 500 µl of 70% ethanol and again centrifuged at 12,000 rpm for 10 min. Finally, the pellet was re-dissolved in 50 µl of 1× TE buffer and stored at −20°Cuntil use.

3.2.8.10 Size screening for recombinant plasmids

Positive clones containing ITS products of appropriate size (700 bp) were identified by PCR screening. The ITS insert was either re-amplified from isolated plasmid DNA using M13-forward (5'-GTAAAACGACGGCCAGT-3') and M13-reverse (5'-CAGGAAACAGCTATGAC-3') plasmid primers or directly from single bacterial colony using colony PCR.

a) Re-amplification of insert using vector specific primers

Plasmid DNA was used as template DNA and 25 µl of PCR reaction contained 50 ng plasmid DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer (M13-forward and M13-reverse) and 2.5 units of *i*-taq^{TM} DNA polymerase (iNtRON Biotechnology, Korea). Amplification reactions were performed with initial denaturation of 3 min at 95ºC, followed by 29 cycles of 1 min at 94ºC, 1 min at 55ºC, and 1.5 min at 72ºC and a final extension of 7 min at 72ºC.

b) Colony PCR

Single bacterial colony was used as template DNA to identify the clones containing ITS inserts of interest i.e. approximately 700 bp in size. PCR was performed in a volume of 25 µl consisting of single bacterial colony as template DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer (ITS1&ITS4) and 2.5 units of *i*-taq^{TM} DNA polymerase (iNtRON Biotechnology, Korea). The thermal cycling conditions were initial denaturation of 3 min at 95ºC, followed by 29 cycles of 30 sec at 94ºC, 30 sec at 50ºC, and 45 sec at 72ºC and a final extension of 7 min at 72ºC.

The amplicons were checked by agarose gel (1.5% w/v) electrophoresis. The clones containing the ITS insert of appropriate size were considered as positive and selected for sequencing purpose.

3.2.8.11 Sequencing

The ITS inserts from positive clones were sequenced at DNA Sequencing Facility at UDSC, Department of Biochemistry, South Campus, University of Delhi, New Delhi, India. The recombinant plasmids were sequenced for the both strands using M13-forward (5'-GTAAAACGACGGCCAGT-3') and M13-reverse (5'-

CAGGAAACAGCTATGAC-3') plasmid primers for pTZ57R/T vectors. Sequencing was performed with an Applied Biosystems automated sequencer using chain termination method (Sanger et al. 1977).

3.2.8.12 Phylogenetic analysis

The ITS sequences obtained in the present study were compared to those in GenBank database using the nucleotide BLAST search algorithm (Altschul et al. 1997). Based on the BLAST results and the outcomes of recent phylogenetic study on *Suillus* (Bruns et al. 2010), closely related *Suillus* sequences were retrieved from GenBank for phylogenetic analysis. In case of large number of homologous sequences for a particular species, only a subset was selected. The sequences were aligned using MAFFT ver. 7.0 (Katoh and Standley 2013) along with our sequences and edited with BioEdit 5.0.6 (Hall 1999). Phylogenetic analysis was performed using Bayesian Inference (BI). The BI was performed with MrBayes v.3.2.2 (Ronquist et al. 2012) with two parallel runs of four incrementally heated simultaneous Monte Carlo Markov Chains over 1 million generations. The sample frequency was set to 100, resulting in an overall sampling of 10,000 trees and the first 2,500 trees were discarded as “burn-in” (0.25). For the remaining trees, a majority rule consensus tree was computed to obtain estimates for Bayesian posterior probabilities (BPP). All the sequences obtained in the present study were deposited in GenBank (Appendix II).

3.3 Physiological characterization and *in vitro* evaluation of *Suillus* isolates

3.3.1 Radial growth and biomass yield

Growth of *Suillus* cultures isolated in the present study 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 25ºC 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 28 days at 25^{o}C in dark conditions. The mycelia were harvested by filtration through pre weighed filter papers (Whatman No. 1) and culture filtrates collected were stored at 4^{o}C for enzyme assays. The mycelia were washed with 3 volumes of distilled water and recovered biomasses were dried at 70^{o}C until constant weight was achieved.

3.3.2 Extracellular Enzyme activities

All the enzyme assays were performed with the culture filtrates stored at 4^{o}C (Section 3.3.1) within one week from the harvesting time.

3.3.2.1 Acid phosphatase activity (Tibbett et al. 1998)

Reagents

1. **5× modified universal buffer (MUB, Stock buffer):**

   MUB stock buffer (Skujins et al. 1962) was prepared by adding 3.025 g of tris (hydroxymethyl) amino methane, 2.9 g of maleic acid, 3.5 g of citric acid and 1.57 g of boric acid to 122 ml of 1 N NaOH solution. Final volume was made up to 250 ml with distilled water and stored at 4ºC.

2. **1× modified universal buffer (MUB, Working buffer):**

   MUB working buffer was prepared by adjusting the pH of 50 ml of a MUB stock buffer with 0.1 N HCl to pH 5.5 and diluting it with distilled water to a final volume of 250 ml.

3. **0.115 M p-nitrophenyl phosphate solution:**

   4.268 gm of disodium p-nitrophenyl phosphate hexahydrate was dissolved in 100 ml of pH adjusted MUB working buffer (pH 5.5). p-nitrophenyl phosphate substrate was stored at 4°C and used within 10 days.

4. **1 N NaOH:**

   40 g of NaOH was dissolved in 800 ml of distilled water and the final volume was made up to 1 l.

5. **p-nitrophenol (pNP):**

   1 mg/ml solution was prepared in 1× modified universal buffer (pH 5.5).

Procedure

1. 1 ml aliquots of culture filtrates were taken in test tubes containing 4 ml of 1× modified universal buffer (1× MUB buffer).
2. After incubating tubes at 30°C for 5 min, 1 ml of filter sterilized 0.115M p-nitrophenyl phosphate solution was added to the tubes.
3. The tubes were capped with screw caps, vortexed for few seconds and then incubated in dark at 30°C for 1 h in a water bath.
4. Reactions were stopped by adding 4 ml of 1 N NaOH to the assay mixtures.
5. Mixtures were vortexed and filtered through whatman filter paper no. 2.
6. The filtrate was taken in glass cuvette and the absorbance of liberated p-nitrophenol (pNP) was measured at 410 nm, against a range of standards (0–50 µg/ml) made

by diluting pNP (1 mg/ml) in equal volumes of 1× modified universal buffer and 1 N NaOH.

7. One unit (1U) acid phosphatase activity was expressed as the amount of enzyme that released 1 µM of p-nitrophenol in the filtrate from the p-nitrophenyl phosphate substrate per hour per gram of mycelial dry weight at pH 5.5 and temperature 30°C.
8. Controls were performed with the same procedure but the substrate (1 ml of 0.115 M p-nitrophenyl phosphate) was added after the addition of 1 N NaOH (i.e. immediately before filtration).

3.3.2.2 Phytase activity

Reagents

1. **Substrate solution:** 2.5 mM sodium phytate in 0.2 M sodium acetate buffer of pH 5.5.
2. **Color stop solution:** 10 mM ammonium molybdate: 5 N sulphuric acid: acetone **(**1:1:2, v/v/v).
3. **1 M citric acid:** 19.21 g of citric acid was dissolved in distilled water and final volume made up to 100ml.
4. **Standard KH_2PO_4 solution:** 1 mg/ml stock.

Procedure

1. For phytase determination, 500 µl of substrate solution (2.5 mM sodium phytate dissolved in 0.2 M sodium acetate buffer of pH 5.5) was taken in a test tube.
2. The enzymatic reaction was started by addition of 500 µl of the enzyme sample (here culture filtrates) to the assay mixture.

3. After an incubation of 20 min at 30°C, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method (Heinonen and Lahti 1981).
4. The reaction was stopped by adding a freshly prepared ice cold color stop solution (2 ml) of 10 mM ammonium molybdate: 5 N sulphuric acid: acetone **(**1:1:2, v/v/v). This was followed by addition of 1 M citric acid (100 µl) to the assay mixture.
5. The final assay mixture was centrifuged to remove any cloudiness and absorbance was taken at 380 nm.
6. Blanks were run by adding the substrate solution after color stop solution.
7. In order to calculate the phytase activity a standard curve was plotted over a range of 10 to 50 µg/ml inorganic phosphate.
8. One unit (1U) phytase activity was defined as the activity that released 1 µM of inorganic phosphate from sodium phytate per hour per gm (Mycelial dry weight) at pH 5.5 and temperature 30°C.

3.3.2.3 Protease activity (Tibbett et al. 1999)

Reagents

1. **Citrate-phosphate buffer (pH 5.5)**
2. **Substrate solution:** 1 mg/ml fluorescein isothiocyanate labeled bovine serum albumen (FITC-BSA)
3. **10% (w/v) trichloroacetic acid (TCA):** 10 gm of trichloroacetic acid was dissolved in 70 ml distilled water and final volume was made up to 100 ml.
4. **0.2 M sodium borate buffer (pH 9.2)**
5. **Protease from *Aspergillus saitoi*:** 0.6 unit/mg stock.

Procedure

1. 1 ml of culture filtrates were taken in test tubes containing 1 ml of citrate phosphate buffer (pH 5.5) and kept at 30°C for 2 min.
2. To start the reaction 50 µl of substrate solution was added to each tube and incubated in a water bath at assay temperature for 2 h.
3. After incubation, 400 µl aliquots of assay mixtures were transferred to centrifuge tubes followed by addition of 1.6 ml 10% (w/v) trichloroacetic acid to stop the reaction.
4. Tubes were kept overnight at 4°C to precipitate the proteins. Insoluble protein contents were then separated by centrifugation at 3000 rpm for 7 min and supernatants were transferred to new collection tubes.
5. For fluorescence measurements, 100 µl of supernatant taken in an auto sampler vial was mixed with 2 ml of 0.2 M sodium borate buffer (pH 9.2) and fluorescence was read on a Turner Biosystems spectroflourimeter at 495 nm excitation and 525 nm emission wavelength (λ_{max} for FITC).
6. A standard curve of fluorescence was prepared with *Aspergillus saitoi* protease enzyme over a range of 0.009–0.036 protease units. One protease unit equivalents (PUE) will hydrolyze casein to produce color equivalent to 1.0 µM of tyrosine per min under assay conditions.
7. Controls, without culture filtrates, were always run in parallel.

3.3.2.4 Chitinase activity

Reagents

1. **2% (w/v) colloidal chitin:** 2 g of chitin was dissolved in 50 mM acetate buffer (pH 5.5) and final volume made up to 100 ml. The suspension was left overnight under shaking condition at 4°C for complete dissolution.
2. **50 mM acetate buffer (pH 5.5)**
3. **Dinitrosalicylic acid (DNS) reagent:** DNS reagent was prepared by adding 10 g of 3,5-dinitrosalicylic acid, 2 g of phenol, 0.5 g of sodium sulphite (Na_2SO_3) and 10 g of NaOH in 800 ml of distilled water and final volume was made up to 1 l. The reagent was stored at 4°C in an amber colored bottle.
4. **40% (w/v) potassium sodium tartrate:** 40 g of Rochelle salt (potassium sodium tartrate) was dissolved in 70 ml distilled water and final volume was made up to 100 ml.
5. **Standard N-acetyl-D-glucosamine solution:** 1 mg/ml stock.

Procedure

1. The assay mixture consisting of 2.9 ml of 2% (w/v) colloidal chitin in 50 mM acetate buffer (pH 5.5) and 0.1 ml of fungal filtrate was incubated at 30°C for 10 min.
2. The amount of N-acetyl-D-glucosamine released during incubation was evaluated by the dinitrosalicylic acid (DNS) method (Miller 1959). Briefly, 3 ml of DNS reagent was added to the reaction mixture and incubated at 90°C for 15 min.
3. After incubation, 1 ml of 40% (w/v) potassium sodium tartrate solution was added immediately to stabilize the color.

4. The mixture was cooled to room temperature, centrifuged to remove insoluble chitin, and the color intensity of resulting supernatant was estimated spectrophotometrically at 575 nm.
5. To calculate the chitinase activity a calibration curve was plotted over a range of 50–250 µg/ml N-acetyl-D-glucosamine.
6. One unit (1U) of chitinase activity corresponds to the amount of enzyme that catalyzed the release of 1 µM of N-acetyl-D-glucosamine from substrate per min per gm of mycelial dry weight under the assay conditions described.
7. Every time, a control without culture filtrate was run in parallel.

3.3.3 Effect of *Suillus* isolates on growth and nutrient contents of *Pinus wallichiana* seedlings

3.3.3.1 *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. In brief, *P. wallichiana* seeds were washed with tap water followed by washing with distilled water and finally surface sterilized in 30% H_2O_2 (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 25ºC for two weeks. Pre-germinated seedlings (1–2 cm root length) were transferred into the tubes containing one month grown *Suillus* isolates. For this purpose, tubes (50 cm^3) were filled with peat, vermiculite (1:10, v/v) mixture and supplemented with 15 ml of liquid Malt extract (2% w/v, pH 5.5) 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 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 µM photon m^{-2} s^{-1} light. After 4 months of growth period, shoot height, root length, seedling’s fresh weight, dry weight and nutrient contents were measured to study the effect of different *Suillus* isolates on pine growth.

3.3.3.2 Influence on 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 a stereomicroscope for each mycorrhizal treatment.

3.3.3.3 Influence on nutrients content of *P. wallichiana* seedlings

Nutrients content, such as N, P, K, Mg and Ca, in seedlings shoots were estimated. The nitrogen content was determined by the Kjeldahl method (Piper 1960), total P by colorimetric method (Kitson and Mellon 1944), and K, Mg and Ca by atomic absorption spectroscopy.

3.3.3.3.1 Total nitrogen in plant samples

The quantity of total nitrogen of the soil was determined by Kjeldahl method as described by Piper (1960).

Reagents

1. **Concentrated sulphuric acid (H_2SO_4)**
2. **0.02 N H_2SO_4:** 0.543 ml of concentrated sulphuric acid (purity 98 %, density 1.84 gm/ml) was added to distilled water and final volume made to 1 l.
3. **50% (w/v) sodium hydroxide:** 20 g of sodium hydroxide pellets were dissolved in distilled water and final volume made to 100 ml.
4. **4% (w/v) boric acid:** 4 g of boric was dissolved in distilled water and final volume was made up to 100 ml.
5. **Sulphuric-salicylic acid:** 1 g of salicylic acid was mixed with 30 ml of sulphuric acid.
6. **Sodium thiosulphate**
7. **Mixed indicator:** 0.066 g of methyl red and 0.099 g of bromo-cresol green was dissolved in 100 ml of ethyl alcohol.
8. **Digestion mixture:** 10 g HgO, 5 g $CuSO_4$ and 100 g K_2SO_4 (2:1:20).

Procedure

1. 5 g of plant sample was taken in a Kjeldahl flask and mixed with sulphuric-salicylic acid followed by addition of 5 g sodium thiosulphate. The mixture was heated for 5 min and cooled down to room temperature.
2. Then 10 g of digestion mixture was added to the flask, contents were mixed well and flask was kept at 100°C for 2 h in a digestion chamber.

3. The color change was observed carefully that turns from dark brown to greenish white, after which the contents were cooled and 300 ml distilled water was added to the flask.
4. For distillation purpose, 20 ml of the digested sample was mixed with 15–20 ml of 50% NaOH and glass beads in a distillation flask through the open end of the condenser attachment, which subsequently was closed with a stopper. A continuous water flow was maintained through the condenser.
5. The distillate was collected in a beaker containing 15 ml of 4 % boric acid and 2 drops of mixed indicator.
6. The distillate was titrated against 0.02 N H_2SO_4 until end point was observed (color changed from green to pink).

Calculation

$$\text{Total nitrogen (\%)} = \frac{(\text{T} - \text{B}) \times \text{Normality of } H_2SO_4 \times 1.4 \ \times 300}{\text{Weight of sample (g)}}$$

Where, T is titer value for sample and B for blank

3.3.3.3.2 Other Elemental analysis of plant samples

a) HNO_3/ $HClO_4$ digestion

For the release of mineral elements from plant sample, di-acid (HNO_3/$HClO_4$) digestion was performed.

Reagents

1. **Concentrated nitric acid (HNO_3) and perchloric acid ($HClO_4$)**
2. **50% (v/v) diluted hydrochloric acid (HCl):** HCl and water in 1:1 ratio.

Procedure

1. 1 g sample of dried plant sample was taken in a digestion flask and 20 ml of concentrated HNO_3 and $HClO_4$ mixture in 3:1 ratio was added to the sample.
2. The sample was digested on an electric heater for the first one hour at 100 °C in an acid proof digestion chamber having fume exhaust system.
3. Heating temperature was then raised gradually to about 180 °C and continued the digestion until the contents become colorless and only white fumes appeared.
4. The acid contents were reduced till whitish dry matter remained in the digestion flask.
5. Allowed it to cool at room temperature after removing it from the heating mental and then dissolved in 50 % diluted HCl and filtered through whatman filter paper no. 42.
6. 2–3 washings with 50 % diluted HCl were given and final volume was made to 50 ml with 50 % diluted HCl.
7. Total phosphorus in the sample filtrates was determined by colorimetric estimation method. Other elements, such as Ca, Mg and K were analyzed through atomic absorption spectrophotometer (GBC 932AA, GBC Scientific Equipment Pvt. Ltd., USA).

b) Total phosphorus in plant samples (Kitson and Mellon 1944)

To determine phosphorus content in the plant samples, molybdo-vanadophosphoric acid method was used as described by Kitson and Mellon (1944).

Reagents

1. **Vanadomolybdate solution:**

   Solution A - 25 g of ammonium molybdate tetrahydrate [$(NH_4)_6 Mo_7O_{24}.4H_2O$] was dissolved in 300 ml of warm water and cooled at room temperature.

   Solution B - 1.25 g of ammonium metavanadate (NH_4VO_3) was dissolved in 300 ml of boiling water. The solution was cooled, followed by addition of 250 ml concentrated HNO_3 and then cooled again. Solution A was mixed gently with solution B and final volume was made up to 1 l in a volumetric flask.

2. **Phosphorus stock standard solution (50 mg/l P):**

   Dry a small amount of potassium dihydrogen phosphate (KH_2PO_4) at 105°C to constant weight and dissolve 219.5 mg of dried salt in about 500 ml of distilled water in a 1 l volumetric flask. Add 25 ml of 7 N H_2SO_4 and dilute to the mark. 4 to 5 drops of toluene were added to prevent any microbial activity.

Procedure

1. 10 ml of acid digested filtrate of plant sample was taken in a 50 ml volumetric flask to which 10 ml of the vanadomolybdate solution was added and then diluted to 50 ml.
2. The contents were mixed well and absorbance was taken after 10 min at 420 nm.
3. To prepare standards, 0, 1, 2, 3, 4 and 5 ml of 50 mg/l stock phosphorus solution were taken in 50 ml volumetric flasks and the color was developed as mentioned above.
4. Spectrophotometer was calibrated with known phosphorus concentrations and then used to read the absorbance of the samples.

Calculation

$$\text{P (mg/kg)} = \frac{\text{Volume made up after digestion (ml)}}{\text{Weight of sample (g)}} \times \frac{50}{\text{Volume of the filtrate used to develop color (ml)}} \times \text{P(mg/l)}$$

3.4 Optimization of culture conditions for mass inoculum production of selected *Suillus* species

For optimization of culture conditions, radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06 was studied. For this purpose, freshly grown fungal colonies were established on 90 mm malt extract agar (2% w/v) plates and grown for 3 weeks. From these agar plates, single agar plug (approximately 6 mm diameter) was placed over each media plate to be optimized (three replicates for each isolate). The plates were incubated for 28 days at 25°C in dark conditions. The radial growth was studied after 4 weeks by measuring the colony diameter.

3.4.1 Medium

To select an appropriate medium for the growth of *S. indicus* SNW02 and *S. sibiricus* SNW06, three different basic fungal media [Modified Melin-Norkrans (MMN) agar media, Malt Extract (ME) agar (2% w/v) media and Potato dextrose agar (PDA) media, see Appendix I] were chosen. The pH was adjusted to 5.5 and the fungal colonies were grown at 25°C for 28 days.

3.4.2 Malt Extract concentration

As ME medium was found optimum for growth of *S. indicus* SNW02 and *S. sibiricus* SNW06, different concentrations of Malt extract (pH 5.5) ranging from 1–20 g/l were evaluated to achieve the best growth. The cultures were grown for 28 days at 25°C.

3.4.3 Effect of various carbon sources on the radial growth

In further optimization, 5 g/l ME and 2.5 g/l ME agar plates were used as they emerged as the optimum ME concentrations for growth enhancement for *S. indicus* SNW02 and *S. sibiricus* SNW06 isolates, respectively. To study the effect of carbon sources, different carbon sources (fructose, galactose, glucose, inositol, lactose, maltose, mannitol, sucrose and sorbitol) were added to the medium. The concentration of each carbon source was adjusted so that the amount of carbon in each medium equals the amount of carbon in medium when supplemented with 10 g/l of glucose. The pH of the medium was adjusted to 5.5 and the cultures were grown for 28 days at 25°C.

3.4.4 Varying concentration of glucose

As glucose was among the one of the carbon sources found optimum for the growth of *S. indicus* SNW02 and *S. sibiricus* SNW06, glucose concentrations were further optimized for these isolates by using different concentrations (5, 10, 15, 20, 25 and 30 g/l) in respective media for both the isolates.

3.4.5 Effect of various nitrogen sources on the radial growth

Effect of different nitrogen sources was studied by supplementing the medium with various nitrogen sources (alanine, arginine, di-ammonium hydrogen phosphate, ammonium tartrate and potassium nitrate). The amount of each nitrogen source added to the medium was adjusted so that the concentration of nitrogen in each medium equals the concentration of nitrogen in medium when supplemented with 10 g/l of di-ammonium hydrogen phosphate. Glucose was added to the respective media (as 20 g/l glucose was found to be optimum for *S. indicus* SNW02 and 15 g/l glucose for *S.*

sibiricus SNW06, respectively). The pH of the medium was adjusted to 5.5 and incubation was carried out at 25°C for 28 days.

3.4.6 di-ammonium hydrogen phosphate concentration

As di-ammonium hydrogen phosphate was found to be optimum nitrogen source for growth of *Suillus* isolates, its concentration was further optimized for these isolates by varying its concentration (2.5, 5, 10, and 20 g/l) in respective media.

3.4.7 Effect of varying concentrations of Adenosine

Adenosine has been shown to promote mycelial growth of *Suillus luteus* as reported by Zhang et al. (2010). In the present study, varying concentrations (0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 g/l) of adenosine were evaluated to enhance the growth of *S. indicus* SNW02 and *S. sibiricus* SNW06. Media optimized so far for both the isolates were supplemented with the different concentrations of adenosine, pH set to 5.5 and incubated at 25°C for 28 days.

3.3.8 Temperature

To 90 mm agar plates of optimized media (OM1 for *S. indicus* SNW02 isolate and OM2 for *S. sibiricus* SNW06 isolate), a single fungal colony of corresponding isolate was transferred aseptically and incubated at 15°C, 20°C, 25°C, 30°C, 35°C and 40°C for 28 days in dark. The radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06 at different temperatures was recorded as described in section 3.4.

3.4.9 pH

The fungal isolate *S. indicus* SNW02 and *S. sibiricus* SNW06 were grown at different pH (4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5) in medium OM1 and OM2, respectively. To observe the effect of pH on the fungal growth, the plates inoculated with *S. indicus*

SNW02 were incubated at temperature 25°C (as growth was maximum at 25°C) and the plates inoculated with *S. sibiricus* SNW06 were incubated at 30°C (as growth was maximum at 30°C). The radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06 at different pH was recorded as described in section 3.4.

3.4.10 Biomass yield of selected *Suillus* species in optimized media

Further, both the selected isolates were grown in optimized broth media under optimized culture conditions to study the effect of optimized media on enhancement of final biomass yield. *S. indicus* SNW02 was grown in 25 ml of OM1 broth medium (pH 5.0) at 25°C and *S. sibiricus* SNW06 in 25 ml of OM2 broth medium (pH 6.0) at 30°C. After incubation for four weeks, mycelia were harvested by filtration through pre weighed filter papers (Whatman No. 1), washed with 3 volumes of distilled water and oven dried at 70°C till constant weight.

3.5 Influence of selected *Suillus* species and biochar amendments on plant growth and rhizosphere properties

3.5.1 Biochar preparation

Biochar was prepared by pyrolysis of locally collected *P. wallichiana* needles at 650°C. Before adding to the soil, biochar obtained was grinded to fine powder and then applied to the sieved soil at the rate of 0 and 2% (w/w).

3.5.2 Inoculum production of selected *Suillus* isolates for field experiments

The inocula were prepared for both the selected *Suillus* isolates in optimized media for these isolates (OM1 for *S. indicus* SNW02 isolate and OM2 for *S. sibiricus* SNW06 isolate). In brief, two mycelia-plugs (6 mm diameter) cut from the margin of three weeks old fungal colony were inoculated in each Erlenmeyer flask and grown at 25°C.

After incubation for 4 weeks, the media contents were decanted off and mycelia were washed 2–3 times with sterile distilled water under sterile conditions. Finally, the mycelia were homogenized aseptically, re-suspended in cold sterile water to obtain 5 mg/ml (dry weight) mycelial suspension and stored at 4°C until use (not more than one month).

3.5.3 Nursery trial and mycorrhizal inoculation of pine seedlings

Meanwhile, a nursery was established in February 2013 at Deeb (1830 m elevation), Kumarsain (31.32°N 77.45°E), Shimla, Himachal Pradesh, India. The forest soil (5–15 cm depth) was collected from an established *P. wallichiana* stand, located 500 m away from the nursery site, after removing the upper forest floor. The soil was sieved through 2 mm net, filled in Polypropylene (PP) bags (17.5×12.5 cm) and then sterilized by autoclaving three times for one hour consecutively for three days.

Prior to sowing, *P. wallichiana* seeds were washed with tap water, surface sterilized in 30% H_2O_2 (v/v) for 25 min followed by washing two times with sterilized distilled water and sown in PP bags (2–3 per bag). After germination of seeds in first week of April 2013, each bag was observed and pruned to one seedling per bag. For inoculations, seedlings were uprooted and 7 ml of homogenized mycelial suspension was dispensed into the rhizosphere zone followed by replanted the seedlings back to the same bags. In total, eighteen seedlings were inoculated with selected *Suillus* species for each biochar treatment and same numbers of seedlings were kept as control. The seedlings were grown under open field conditions and watered by sprinkling once a week.

3.5.4 Experimental design and morphotyping of ectomycorrhizae

The experiment was set up in randomized block design manner with six treatments and four replication blocks. Each replication block consisted of six seedlings for each treatment. Thus, total of 24 seedlings were maintained for each treatment. The six treatments established were— control soil (C), *S. indicus* SNW02, *S. sibiricus* SNW06, 2% Biochar (BC), 2% Biochar + *S. indicus* SNW02 and 2% Biochar + *S. sibiricus* SNW06. The destructive sampling was carried out in early January (eleven months after sowing or nine months after inoculation). In total, seven plants per treatment were randomly selected from different blocks (total of 24 plants per treatment) for sampling of seedlings and rhizosphere soils.

Ectomycorrhizal roots were washed gently in tap water and ectomycorrhizae formed were observed under a Nikon SMZ800 stereomicroscope (Nikon, Japan). Images were captured with a Nikon Digital Sight DS-5MC camera. Mantle preparations of fresh mycorrhizal roots were fixed on slides with Lacto-glycerin or Hardy Diagnostics BlueMount™ and mantle types, emanating elements, and other anatomical observations were documented using an Olympus light microscope (Olympus, Japan) with the aid of a camera lucida. The general morphological and microscopic descriptions of the ectomycorrhizae followed the methodology and terminology of Agerer (1991).

3.5.5 Plant and soil analytical procedures

3.5.5.1 Determination of soil pH

Procedure

The pH of soil/biochar water suspension was determined with a pH meter as described by Jackson (1967).

1. 10 g of air dried soil sample sieved through 2 mm net was mixed with 50 ml of distilled water (1:5) taken in a 250 ml flask.
2. The suspension was stirred continuously for 5 min using a glass rod and then kept for shaking at 130 rpm for 30 min.
3. Finally, the suspension was allowed to stand for next 1 h and the supernatant collected was used for pH detection.
4. For pH determination, the pH meter was switched on and pH electrode was calibrated with three standard buffer solutions of known pH (pH 7.0, pH 4.0 and pH 9.2).
5. After calibration, electrode was washed with distilled water, dipped into supernatant solution and pH was recorded.
6. The electrode was rinsed with distilled water and wiped with tissue paper every time before taking reading of a new soil sample.

3.5.5.2 Determination of soil cation exchange capacity (CEC)

The cation exchange capacity (CEC) was determined using ammonium acetate method described by Chapman (1965).

Reagents

1. **1 M ammonium acetate (NH_4OAc) solution:** In a fume hood, 57 ml of glacial acetic acid (99.5%) was diluted with distilled H_2O to about 800 ml in a 1 l volumetric flask. Then added 68 ml of concentrated ammonium hydroxide (~58% NH_4OH), mixed well and cooled to room temperature. Adjusted pH to 7.0 by adding NH_4OH dropwise and finally diluted to 1 l.
2. **1 M KCl extracting solution:** Dissolved 74.5 g of potassium chloride (KCl) in distilled H_2O and final volume made up to 1 l.
3. **Ethanol (95%)**

Procedure

1. 25.0 g of air dried soil/biochar sieved through 2 mm net was taken into a 500 ml Erlenmeyer flask.
2. Added 125 ml of the 1 M NH_4OAc solution mixed thoroughly by gentle shaking and kept for 16 h or overnight.
3. A whatmann filter paper no. 42 was fitted to a 5.5 cm Buchner funnel and moistened with a minimum amount of NH_4OAc. Buchner funnel was inserted to a filter flask (Buchner flask) and a light suction was applied to the side arm of the filter flask using a vaccum pump. The overnight kept soil-NH_4OAc mixture was transferred to the funnel and filtered (Re-filter the filtrate through the soil, if filtrate is not clear).
4. The soil was leached four times with additional 25 ml of the NH_4OAc (Don’t let the soil dry between subsequent filtration).

5. The filtrate was discarded and the soil was washed eight times with 95% ethanol (enough to cover the soil surface) to remove excess saturating NH_4OAc solution. The filtrate was discarded and the filter flask was replaced with fresh one.
6. To extract the adsorbed NH_4, the soil was leached eight times with 25 ml of 1 M KCl. The filtrate was transferred to a 250 ml volumetric flask and made up the volume using 1 M KCl.
7. Finally, the concentration of NH_4-N in the KCl extracts and the original KCl extracting solution (blank) were analyzed either by distillation or by calorimetric methods.

Calculations

$$\mathrm{CEC}\left(\frac{\mathrm{cmol}}{\mathrm{kg}}\right) = \frac{\mathrm{mg\ NH_4\ in\ extract} - \mathrm{mg\ NH_4\ in\ blank}}{18}$$

3.5.5.3 Total organic carbon (TOC)

The percentage of total organic carbon in the soil was determined according to Walkley and Black (1934).

Reagents

1. 1 N potassium dichromate: 49.04 g $K_2Cr_2O_7$ was dissolved in 800 ml of distilled water and final volume was made up to 1 l.

2. 0.5 N ferrous ammonium sulphate: 198 g ferrous ammonium sulphate was dissolved in 800 ml of distilled water and final volume was made up to 1 l.

3. Diphenyl amine indicator: 500 mg of diphenyl amine indicator (DPA) was dissolved in a mixture of 20 ml water and 100 ml conc. H_2SO_4.

4. Concentrated Sulphuric acid (H_2SO_4)

5. Orthophosphoric acid (85%)

6. Sodium fluoride (NaF)

Procedure

1. Soil/biochar samples were ground and sieved through 2 mm mesh. 1 g of a sample was taken in a conical flask (500 ml) and 10 ml of 1 N $K_2Cr_2O_7$ was added with a measuring cylinder.
2. The flask was swirled thoroughly to mix the soil and reagent, completely.
3. To the mixture, 20 ml of concentrated H_2SO_4 was poured gently along the flask wall using a measuring cylinder and the flask was kept aside for 30 min at room temperature.
4. Then 200 ml of distilled water was added to the flask followed by addition of 10 ml of orthophosphoric acid (85 %), 0.5 g of NaF and 1 ml of diphenylamine indicator.
5. Finally, the content was titrated with freshly prepared 0.5 N ferrous ammonium sulphate till the end point was achieved i.e. from blue violet to green. A blank titration without soil was also carried at the beginning.

Calculation

$$\text{Organic carbon (\%)} = \frac{10(\mathrm{B} - \mathrm{T}) \times 0.003 \times 100}{\mathrm{B} \times \text{Initial Soil weight (g)}}$$

Where,

B is volume of ferrous ammonium sulphate solution used for blank titration and T is volume of ferrous ammonium sulphate solution used for soil sample titration.

3.5.5.4 Available phosphorus (P)

Available phosphorus in the soil and pine needle biochar was estimated by the sodium bicarbonate method (Olsen et al. 1954).

Reagents

1. **0.5 M $NaHCO_3$ extracting solution:** 84 g of sodium bicarbonate was added in distilled water and volume was made up to 2 l. The pH was adjusted to 8.5 with 1M or 1N NaOH.
2. **Reagent A:** 12 g ammonium molybdate in 250 ml distilled water and 0.2908 g antimony potassium tartarate in 100 ml distilled water were added to 1000 ml of 2.5 M H_2SO_4, mixed thoroughly and volume was made up to 2 l with distilled water.
3. **Reagent B (freshly prepared):** 1.058 g of ascorbic acid was added in 200 ml of reagent A and mixed.
4. **Sulphuric acid (2.5 M):** 140 ml of conc. H_2SO_4 was diluted to 1 l.
5. **Stock standard P solution (50 ppm):** 0.2917 KH_2PO_4 was dissolved in distilled water to a final volume of 1 l.
6. **Working standard P solution (1 ppm):** 20 ml of 50 ppm solution was diluted to 1 l.

Procedure

1. 2.5 g of powdered soil/biochar was mixed in 50 ml extracting solution, kept on a shaker for 30 min and then filtered through whatman filter paper no. 42.
2. Pipette 10 ml aliquot of filtrate into a 100 ml beaker.
3. Added 1 ml of 2.5 M H_2SO_4, 15.5 ml of distilled water followed by addition of 8 ml reagent B and volume made up to 50 ml with distilled water.

4. Read the absorbance at a wavelength of 882 nm after 10 min against a method blank.

5. Blank was prepared by taking 10 ml of extracting solution in place of the soil extract.

6. To prepare standard curve, 0, 2, 5, 10, 15 and 20 ml of 1 ppm working standard solution was dispensed in separate 50 ml volumetric flasks. 10 ml of extracting solution, 1.0 ml of 2.5 ml H_2SO_4 and 8 ml Reagent B were added and the volume was made up to 50 ml with distilled water. The P concentrations of these solutions range from 0.04 ppm–0.4 ppm. After 10 min, absorbance was read at 882 nm.

Calculation

$$\text{Available P in soil/biochar (ppm)} = \text{P in extract (ppm)} \times 20$$

3.5.5.5. Total nitrogen (Piper 1960)

Total nitrogen content in plant and soil samples was estimated by the Kjeldahl method as described earlier in section 3.3.3.3.1.

3.5.5.6 Total phosphorus and other elemental analysis

a) HNO_3/ $HClO_4$ digestion

For elemental analysis, plant samples were digested using $HNO_3/HClO_4$ digestion method as described earlier in section 3.3.3.3.2a. Soil samples were digested with the same reagents but with some modifications.

1. 1 g of air dried soil sample was weighed and taken in a digestion tube. 10 ml concentrated HNO_3 was added and the contents were digested on electric heater for 1 h at 145°C in acid proof digestion chamber having fume exhaust system.

2. Allowed to cool at room temperature and followed by addition of 10 ml concentrated HNO_3 and 5 ml $HClO_4$. The contents were heated at about 100°C for the first 1 h and then the temperature was raised gradually to about 200°C.
3. The digestion was continued until the contents become colorless and only white fumes appeared.
4. The acid content was reduced till white matter remains left in the digestion tube.
5. Thereafter, the digestion tube was removed from the heating mental and cooled to room temperature. Then 50 % diluted HCl was added to the tube and filtered through whatman filter paper no. 42.
6. 2 or 3 washings with 50 % diluted HCl was given and final volume was made up to 50 ml with diluted 50 % HCl.
7. The filtrates obtained were used to determine total P in plant and soil samples by calorimetric method and K content only in plant samples with an atomic absorption spectrophotometer (GBC 932AA, GBC Scientific Equipment Pvt. Ltd., USA).

b) Total phosphorus in plant samples (Kitson and Mellon 1944)

Total phosphorus content in filtrates of the plant and soil samples was determined as discussed earlier in section 3.3.3.3.2b.

3.5.6 Soil enzyme activities

3.5.6.1 Acid phosphatase activity in soil (Tabatabai and Bremner 1969)

Reagents

1. **5× modified universal buffer (MUB, Stock buffer):**

   MUB stock buffer (Skujins et al. 1962) was prepared by adding 12.10 g of tris (hydroxymethyl) amino methane, 11.60 g of maleic acid, 14.0 g of citric acid and

6.28 g of boric acid to 488 ml of 1 N NaOH solution. Final volume was made up to 1000 ml with distilled water and stored at 4ºC.

2. **1× modified universal buffer (MUB, Working buffer):**

   MUB working buffer was prepared by adjusting the pH of 200 ml of a MUB stock buffer with 0.1 N HCl to pH 5.5 and diluting it with distilled water to a final volume of 1000 ml.

3. **0.115 M p-nitrophenyl phosphate solution:**

   4.268 gm of disodium p-nitrophenyl phosphate hexahydrate was dissolved in 100 ml of pH adjusted MUB working buffer (pH 5.5). p-nitrophenyl phosphate substrate was stored at 4ºC and used within 10 days.

4. **0.5 N NaOH:**

   20 g of NaOH was dissolved in 800 ml of distilled water and the final volume was made up to 1000 ml.

5. **p-nitrophenol (pNP):**

   1 mg/ml solution was prepared in 1× modified universal buffer (pH 5.5).

Procedure

1. 1 g of air dried soil samples were taken in flasks containing 4 ml of 1× modified universal buffer (1× MUB buffer).
2. Acid phosphatase activities were then determined as described earlier in section 3.3.2.1.

3.5.6.2 Dehydrogenase activity (Tabatabai 1994)

Dehydrogenase activity was determined by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenylformazan (TPF).

Reagents

1. **1, 3, 5 triphenyl tetrazolium formazan (TPF) standard (100 µg/ml):** 10 ml of TPF stock solution (1 mg/ml) was dispensed in methanol and final volume was made up to 100 ml in a volumetric flask.
2. **3% 2, 3, 5 triphenyl tetrazolim chloride (TTC):** 3 g of TTC was dissolved in distilled water and volume made up to 100 ml.
3. **Calcium carbonate ($CaCO_3$)**
4. **Methanol**

Procedure

1. A 20 g sample of moist mineral soil was mixed thoroughly with 0.2 g of $CaCO_3$ (100:1) and 6 g of this mixture was transferred into three separate test tubes.
2. 1 ml 3% aqueous TCC solution was added to each tube followed by addition of 2.5 ml of distilled water.
3. The contents were mixed thoroughly with a glass rod and incubated at 37°C for 24 h.
4. Then 10 ml of methanol was added to extract the TPF formed as a result of TTC reduction and subsequently filtered through whatman filter paper no. 1. The filter paper was washed with additional methanol until the reddish color disappeared and the final volume of filtrate was adjusted to 100 ml.
5. The optical density of red colored filtrate was determined at 485 nm with methanol as a blank.
6. To prepare standards, 2, 5, 10, 15 and 20 ml of TPF standard (100 µg/ml) solution were taken in 100 ml volumetric flasks and final volume adjusted with methanol and the color intensity was read at 485 nm.

3.5.6.3 Protease activity

1. **0.1 M Phosphate buffer (pH 7.0)**

2. **0.03 M N-α-benzoyl-L-argininamide (BAA) substrate solution:** 995 mg of N-α-benzoyl-L-argininamide hydrochloride monohydrate was dissolved in distilled water and final volume made up to 100 ml.

3. **Standard ammonium chloride solution:** To prepare stock ammonium chloride (NH_4Cl) solution (100 ppm), 0.3819 g of dried anhydrous NH_4Cl was dissolved in distilled water, and diluted up to 1 l. A standard NH_4Cl solution (5 ppm) was prepared by diluting the 100 ppm NH_4Cl solution 20 times with distilled water.

4. **Sodium phenate solution:** Dissolve 10 g phenol and 1.76 g sodium hydroxide (NaOH) in distilled water and then add 6 ml acetone. Add water to a final volume of 200 ml.

5. **Sodium nitroprusside:** Dissolve 0.15 g sodium nitroprusside in 100 ml distilled water.

6. **Sodium hypochlorite (NaOCl):** 10 % commercial solution. Use within 2 months after bottle is opened.

Procedure

1. To measure protease activity of soil samples, 2 ml of 0.1 M phosphate buffer (pH 7) and 0.5 ml of 0.03 M N-α-benzoyl-L-argininamide (BAA) were added to 500 mg of soil.

2. The mixtures were incubated at 37°C for 90 min and then diluted to 10 ml with distilled water.
3. The ammonium released was estimated using phenate method (Park et al. 2009).
4. In brief, 5–10 ml of soil extracts were added into 50 ml volumetric flask and diluted with distilled water up to 25 ml. To this, 10 ml of sodium phenate, 1 ml of sodium nitroprusside and 5 ml of sodium hypochlorite solution were added and then final volume made upto marked line (50 ml) with distilled water.
5. Contents were mixed and kept as it is at 20–25°C for 30 min. The absorbance was recorded at 630 nm against a blank.
6. The standard curve for ammonia estimation was prepared using 0.1–1 ppm NH_4Cl concentrations prepared from 5 ppm standard NH_4Cl solution.

3.5.6.4 Urease activity

Reagents

1. **0.1 M Phosphate buffer (pH 7.0)**
2. **6.4% (w/v) Urea:** 6.4 g of urea was dissolved in distilled water and volume made up to 100 ml.
3. **Standard ammonium chloride solution:** To prepare stock ammonium chloride (NH_4Cl) solution (100 ppm), 0.3819 g of dried anhydrous NH_4Cl was dissolved in distilled water, and diluted up to 1 l. A standard NH_4Cl solution (5 ppm) was prepared by diluting the 100 ppm NH_4Cl solution 20 times with distilled water.
4. **Sodium phenate solution:** Dissolve 10 g phenol and 1.76 g sodium hydroxide (NaOH) in distilled water and then add 6 ml acetone. Add water to a final volume of 200 ml.

5. **Sodium nitroprusside:** Dissolve 0.15 g sodium nitroprusside in 100 ml distilled water.
6. **Sodium hypochlorite (NaOCl):** 10 % commercial solution. Use within 2 months after bottle is opened.

Procedure

1. To determine urease activity in soil samples, 2 ml of 0.1 M phosphate buffer (pH 7) and 0.5 ml of 6.4% urea were added to 500 mg of soil.
2. The contents were then incubated at 37°C for 90 min.
3. Finally, the volume was made up to 10 ml with distilled water and ammonium released was estimated using phenate method (Park et al. 2009) as described in previous section for protease assay.

3.6 Statistical analysis

Multiple replicates were used for each experiment (three replicates when not mentioned). In nursery trials, plots with different treatments were arranged completely in a randomized block design. 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.

Chapter 4

Results

4.1 Diversity of *Suillus* from northwestern Himalayas

4.1.1 Occurrence of *Suillus* basidiocarps

Various localities of northwestern Himalaya region of India were explored to collect the sporocarps of *Suillus* species. (Table 4.1). In total 40 specimens were collected, out of which 6 were collected from Jammu and Kashmir (J.&K.), 22 from Himachal Pradesh (H.P.) and 12 from Uttarakhand (U.K.) (Table1). It was found that their occurrence prevails from 1300 m [mid hill sub-montane zone (1000–2000 m)] to 2800 m [high hill wet temperate zone (2000–3000 m)]. Generally, they start appearing after first showers of monsoon in the first half of July and flourish well during July–August and even up to first half of September, depending upon the duration of monsoon season. During our collection trips to northwestern Himalayas, *Suillus* species were found mostly in mid monsoon season (15th July–15th August).

4.1.2 Identification of *Suillus* species based on Classical taxonomy

All the specimens collected during our excursion trips were used for taxonomic purpose and the dried reference materials were deposited at the Herbarium of Botany, Department of Botany, Punjabi University (PUN), Patiala, Punjab, India (Table 4.1). On the basis of classical taxonomy of these specimens, they were classified into seven different species of *Suillus*.

Basidiocarps of *Suillus* are usually characterized by viscid and slimy pileal cap, especially under wet conditions. Pileal surface is glabrous to scaly. The hymenophore is

Table 4.1 Different *Suillus* specimens collected from three different states of north western Himalayas, India along with collection site, date of collection and forest type

S. No.	Collection/ Voucher No.	State	District, Sub locality, Altitude	Date of collection	Forest type*
1	SJK01/PUN 5520	Jammu and Kashmir	Ganderbal, Mamar, 1916 m	02/08/2009	PW
	SJK02/PUN 5521		Kulgam, Aharbal, 2266 m	05/08/2009	PW
	SJK03/PUN 5522		Kulgam, Aharbal, 2266 m	05/08/2009	PW
	SJK11/PUN 5523		Udhampur, Kud, 1855 m	18/07/2010	PR
	SJK12/PUN 5524		Anantnag, Pahalgam, 2740 m	20/07/2010	PW
	SJK13/PUN 5525		Anantnag, Kukernag, 2000 m	21/07/2010	PW
2	SUK02/PUN 5526	Uttarakhand	Uttarkashi, Barkot, 1524 m	29/07/2010	PW
	SUK03/PUN 5527		Dehradun, Mussoorie, 1825 m	30/07/2010	PR
	SUK05/PUN 5528		Chamoli, Naagnath, 1615 m	02/08/2010	PW
	SUK06/PUN 5529		Chamoli, Joshimath, 1890 m	04/08/2010	PW
	SUK07/PUN 5530		Chamoli, Gwaldam, 1708 m	06/08/2010	PW
	SUK11/PUN 5531		Nainital, Nainital, 2084 m	20/08/2011	PR
	SUK12/PUN 5532		Nainital, Mukteshwar, 2290 m	21/08/2011	PW
	SUK20/PUN 6569		Uttarkashi, Janki Chatti, 2650 m	11/08/2012	PS
	SUK23/PUN 6570		Uttarkashi, Harsil, 2620 m	13/08/2012	PW
	SUK24/PUN 6571		Tehri Garhwal, Tehri, 1750 m,	16/08/2012	PW
	SUK26/PUN 6572		Tehri Garhwal, Chandrabadni, 2277 m	18/08/2013	CD
	SUK27/PUN 6573		Tehri Garhwal, Dhanaulti, 2286 m	22/08/2013	PW
3	SHP01/PUN 6574	Himachal Pradesh	Shimla, Baghi, 2685 m	25/07/2010	PW
	SHP02/PUN 6575		Shimla, Kharapathar, 2673 m	25/07/2010	PW
	SHP03/PUN 6576		Shimla, Narkanda, 2621 m	27/07/2010	MF (PW&CD)
	SHP04/PUN 5533		Shimla, Oddi, 1850 m	26/07/2011	PW
	SHP05/PUN 6577		Shimla, Shallan, 1900 m	26/07/2011	PW
	SHP07/PUN 6578		Shimla, Kandyali, 2450 m	29/07/2011	MF (PW&CD)
	SHP11/PUN 5534		Mandi, Karsog, 1404 m	05/08/2011	PR
	SHP12/PUN 6579		Mandi, Seri, 2065 m	06/08/2011	PW
	SHP14/PUN 6580		Mandi, Chindi, 1825 m	06/08/2011	PW
	SHP15/PUN 5535		Mandi, Lachkandi, 1829 m	07/08/2011	PW
	SHP21/PUN 5536		Kullu, Dagsari, 2350 m	25/07/2012	CD
	SHP22/PUN 6581		Kullu, Jibhi, 2300 m	25/07/2012	PW
	SHP23/PUN 6582		Kullu, Banogi, 1340 m	26/07/2012	PW
	SHP26/PUN 5537		Mandi, Tikkan, 1829 m	28/07/2012	PW
	SHP27/PUN 5538		Kangra, Palampur, 1472 m	29/07/2012	PR
	SHP28/PUN 5539		Chamba, Tissa, 1870 m	05/08/2012	PW
	SHP30/PUN 6583		Chamba, Bharmour, 2133 m	03/08/2012	PW
	SHP32/PUN 6584		Shimla, Theog, 1965 m	10/07/2013	PW
	SHP34/PUN 6585		Kinnaur, Kalpa, 2960 m	18/07/2013	CD
	SHP35/PUN 6586		Sirmaur, Shillai, 1900 m	28/07/2013	PW
	SHP37/PUN 6587		Sirmaur, Haripurdhar, 2500 m	31/07/2013	CD
	SHP38/PUN 6588		Sirmaur, Tarna, 1969 m	02/08/2013	PW

*PR, PW, CD, PS, and MF stands for *Pinus roxburghii*, *Pinus wallichiana*, *Cedrus deodara*, *Picea smithiana* and mixed forest type, respectively.

tubular with adnate, sudecurrent to decurrent attachment types. Tubes are mostly radially arranged with compound to angular pore mouths. Stipe is central or rarely excentric, mostly with conspicuous glandular dots or smears on its surface that may be absent in some species. Spore deposit varies from light brown through yellow brown to olive shades. Anatomically, the cystidial elements are generally fasciculated into bundles and incrusted with brown coagulated contents. In some species, cystidial or fascicles bases are also incrusted with brown particles. Tube trama consisted of gelatinous divergent hyaline hyphae. In general, clamp connections are not observed in the hyphae of *Suillus* sporocarps. Presence or absence of partial veil on the pileal margin, annulus and glandular dots on the stipe, flesh color change and color of mycelium at stipe base are few important morphological characteristics for species delimitation in the genus *Suillus*.

4.1.3 Taxonomic descriptions

The Genus: *Suillus*

S.F. Gray in "A Natural Arrangement of British Plants" 1821: 646p
Type species: *Suillus luteus* (L.) Roussel (Gray 1821)
Emended by Smith and Thiers in "A contribution toward a monograph of the North American species of *Suillus*" 1964: 116 pp (Smith and Thiers 1964)

1. *Suillus triacicularis* sp. nov.

Suillus triacicularis B. Verma & M.S. Reddy, **sp. nov.** (Figs. 4.1&4.2)

MycoBank no.: MB 807281.

ETYMOLOGY: The specific epithet "*triacicularis*" is derived from Latin and refers to the fact that the new species occurs in association with a 'three-needle' pine.

DIAGNOSIS**:** The species differs from *Suillus granulatus* by the absence of watery green context above the tubes during any stage of development. The absence of cloudy droplets over the tubes when young and the yellow to reddish or orange-yellow pileus color at maturity also distinguish it from *Suillus granulatus*.

TYPE: INDIA. Himachal Pradesh: Mandi, Karsog, 1404 m, 5th August 2011, *B. Verma* (PUN 5534).

DETAILED DESCRIPTION:

Macrocharacters: Basidiocarps 5.0–8.5 cm in size. Pileus 4.0–8.0 cm broad, convex when young, flattened with age; margin regular, strongly incurved when young, irregular and undulating with maturity; pileal veil absent at any stage of development; pileus surface dry to moist, slightly viscid during rainy season, glabrous, yellowish white (1A2) to pale yellow (1A3) when young turning yellow (2A6–2A7) then to reddish or orange yellow with age (4B7–4B8), cuticle fully peeling, umbo absent; scales absent; pileus context concolorous with pileus (1A2–1A3), unchanging, slowly turning light yellow then to yellow (2A4–2A6) on exposure, ≤ 18 mm thick; odor not distinctive and taste mild. Tubes 1–5 mm deep, decurrent, radial, crowded, fully peeling, yellowish grey to greyish yellow (2B2–2B6), unchanging, spotted light brown to brown with age; pore mouths angular, 1–2 per mm. Stipe central, 3.0–6.5 cm long, 8–12 mm thick, tubular and equal in diameter, concolorous with pileus (1A2–1A3), unchanging with age or when bruised, covered with orange red to reddish brown (8A8–8D8) glandular dots and smears throughout but fewer at base; annulus absent; white mycelium at the base. Stipe context pale yellow (1A3), unchanging, solid, becoming hollow with age.

Microcharacters: Spore deposit light brown (6D8), spores 6.2–8.5 × 2.3–3.8 μm (Q=2.0), elongated or oblong in shape, nearly hyaline in KOH, pale yellow in Melzer's reagent, smooth, with granular content inside. Basidia 16.0–24.0 × 4.4–6.2 μm, clavate to cylindrical, granular, 4 spored, hyaline in KOH and yellowish in Melzer's reagent;

sterigmata 1.5–3.9 µm high. Pleurocystidia numerous to abundant, 40.0–62.0 × 6.0–8.0 µm, cylindrical to subclavate, in fascicles or scattered, hyaline with uniform brown coagulated contents in KOH, yellowish with brownish contents in Melzer' reagent. Cheilocystidia 24.5–38.5 × 3.0–9.3 µm, mostly in fascicles, hyaline in KOH, yellowish in Melzer's reagent and brown content absent inside. Caulocystidia 26.0–70.0 × 6.0–9.3 µm in size and almost similar to pleurocystidia. Trama gelatinous and divergent. Clamp connections absent.

Fig. 4.1 *Suillus triacicularis* (basidiocarps): **A.** Young sporocarps with yellowish white to pale yellow pileus; **B.** Mature sporocarps showing yellow to reddish or orange-yellow pileus; **C.** Light brown to brown spotted tubes; **D.** Orange-red to reddish brown glandular dots or smears on stipe. Scale bars: A–D=2 cm

CHEMICAL COLOR REACTIONS: Pileal flesh: 2.5% KOH— reddish, 10% $FeSO_4$— grayish green, 14% ammonia— reddish then to reddish brown, conc. HNO_3— no color reaction. Pileus cuticle: 2.5% KOH— light brown then to blue black, 10% $FeSO_4$—

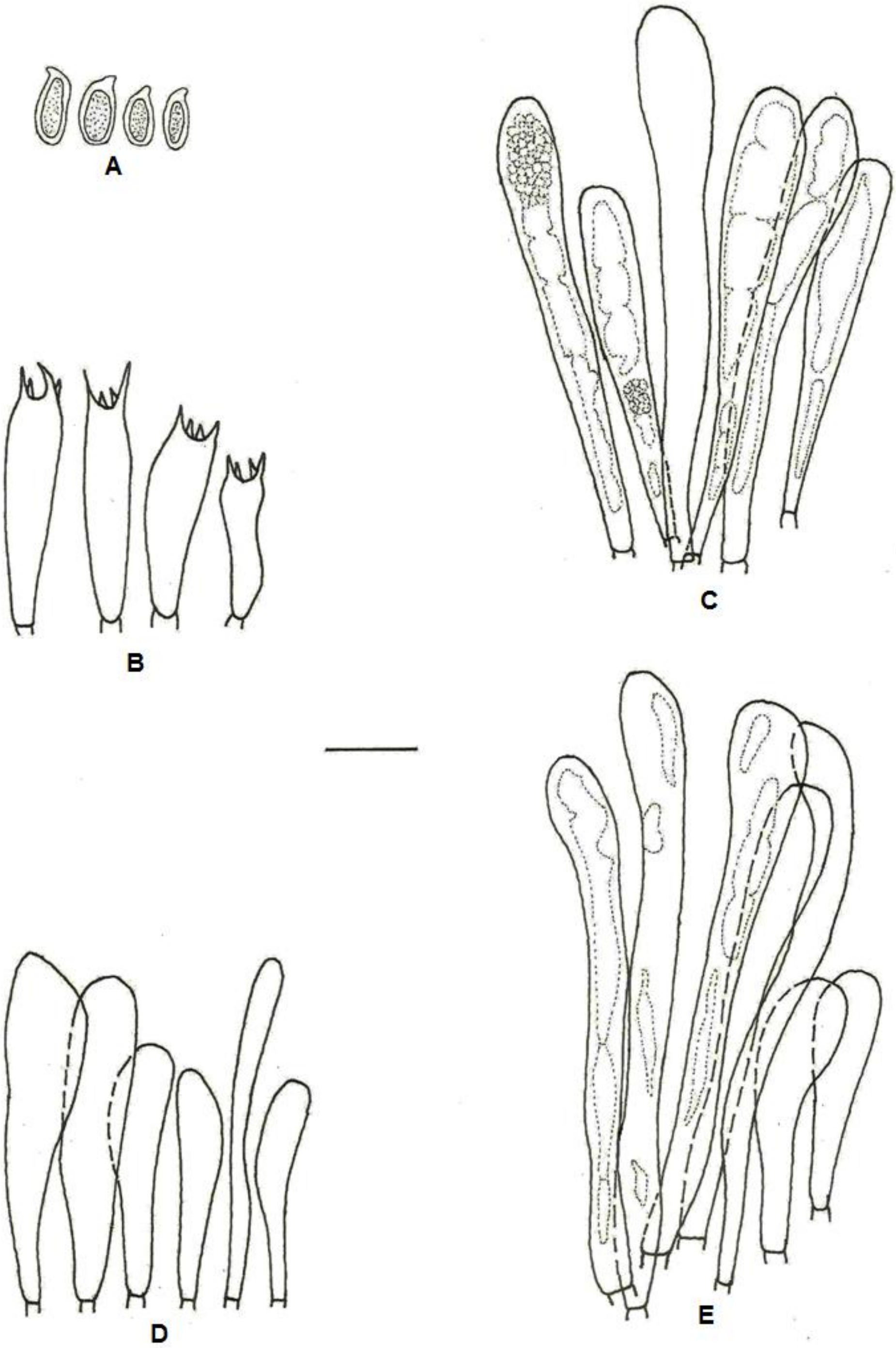


Fig. 4.2 *Suillus triacicularis*, microscopic line drawings: **A.** Basidiospores; **B.** Basidia; **C.** Pleurocystidia; **D.** Cheilocystidia; **E.** Caulocystidia. Scale bar: A–E=10 μm

olive gray, 14% ammonia— brown then to black brown, conc. HNO_3— no color reaction. Baroni (1978) studied the chemical spot tests of 6 genera of *Boletaceae* and found that pink to reddish color reaction of pileal flesh with KOH and ammonia distinguishes *Suillus* species from other *Boletaceae*.

HABITAT AND DISTRIBUTION: *Suillus triacicularis* is found fruiting solitary, scattered or gregarious under *P. roxburghii* trees during the monsoon season. Its distribution ranges with the natural distribution of *P. roxburghii* in the northwestern Himalayas. The occurrence of *S. triacicularis* is rare and the species is seldom found in pure stands of *P. roxburghii* in this geographical region. No records were found from other *Pinaceae* forests during our 5 years of excursions to these forests from 2009 to 2013.

OTHER SPECIMENS EXAMINED: INDIA. Jammu and Kashmir: Udhampur, Kud, 1855 m, 18th July 2010, *B. Verma* (PUN 5523). Himachal Pradesh: Kangra, Palampur, 1472 m, 29th July 2012, *B. Verma* (PUN 5538). Uttarakhand: Dehradun, Mussoorie, 1825 m, 30th July 2010, *B. Verma* (PUN 5527); Nainital, 2084 m, 20th August 2011, *B. Verma* (PUN 5531).

2. *Suillus indicus* sp. nov.

Suillus indicus B. Verma & M.S. Reddy **sp. nov.** (Figs. 4.3&4.4)

MycoBank No.: MB 808527.

ETYMOLOGY: The specific epithet "*indicus*" is derived from Latin with reference to the new species being reported from the Indian region.

DIAGNOSIS: The species is morphologically close to *Suillus decipiens* (Peck) Kuntze and differs mainly by the absence of fibrillose squamules over the stipe. The presence of umbo and less numerous fibrillose squamules over the pileal surface also distinguish it from *S. decipiens*. Microscopically, the species varies from *S. decipiens* by presence of few 2-spored basidia on hymenium and absence of caulocystidia on the stipe surface.

TYPE: INDIA. Himachal Pradesh: Shimla, Narkanda, 2621 m, 27th July 2010, *B. Verma* (PUN 6576).

DETAILED DESCRIPTION:

Macrocharacters: Basidiocarps ranges 4.0–10.0 cm in height. Pileus 3.0–9.0 cm broad, convex when young, flattened or slightly upturned with age; margin regular, appendiculate with white veil fragments (1A1); pileus surface dry to moist, not viscid or slightly viscid only when wet, brownish orange (6C4–6C6) when young, reddish brown (8D7–8D8) with pale yellow ground color in age, low obtuse umbo present and cuticle fully peeling; brownish red (8C7–8C8) to reddish brown (8D7–8D8) fibrillose squamules present throughout the pileal surface. Pileus context ≤ 20 mm thick, soft, pale yellow (2A3) to yellowish (2A6) in age, unchanging or slowly staining pinkish when exposed; taste mild and odor not distinctive. Tubes up to 8 mm deep, decurrent, radial, fully peeling, yellow (3A6–3A7) when young, brownish yellow (5C7–5C8) with age, slowly turning light brown on bruising; pore mouths irregular, 1–3 mm broad. Stipe central, 3.0–8.0 cm long, 1.0 cm–1.2 cm thick, tubular and slightly enlarged downward, typically clavate in young specimens, glandular dots absent, concolorous with the tubes at top and middle and white (1A1) at base; white colored annulus present turning brown with time; mycelium at base white. Stipe context solid, pale yellow (2A3–2A6) and turning dirty brown when exposed.

Microcharacters: Basidiospores cinnamon brown (6D6) in mass, hyaline to pale yellow in KOH, yellowish in Melzer's reagent, smooth with granular content inside, 7.5–11.5 × 3.0–4.5 µm in size and cylindric to subcylindric in shape (Q=2.6). Basidia 17.0–30.0 ×

Fig. 4.3 *Suillus indicus* (basidiocarps): **A.** Young basidiocarp showing umbo and very few appressed fibrillose squamules on the pileus; **B.** Young basidiocarp showing white partial veil and absence of glandular dots; **C.** Mature basidiocarp with appressed fibrillose squamules and a low obtuse umbo on pileal surface; **D.** Stipe with annulus and no glandular dots/smears. Scale bars: A–B = 1 cm, C–D = 2 cm

4.5–6.5 µm, clavate, 4-spored, occasionally 2-spored, hyaline to yellowish in KOH, yellowish with yellowish brown contents in Melzer's reagent; sterigmata 1.5–3.1 µm high. Pleurocystidia 18.0–54.0 × 4.0–8.5 µm, cylindrical to subclavate with blunt to constricted tips, scattered or in fascicles, granular mostly with brown coagulated contents inside, hyaline in KOH, yellowish brown in Melzer's reagent. Cheilocystidia 20.0–62.0 × 3.0–5.0 µm, shape similar to pleurocystidia, granular, slightly yellowish in KOH, brownish in Melzer's reagent, abundant and mostly in fascicles. Caulocystidia absent. Trama divergent and clamp connection absent.

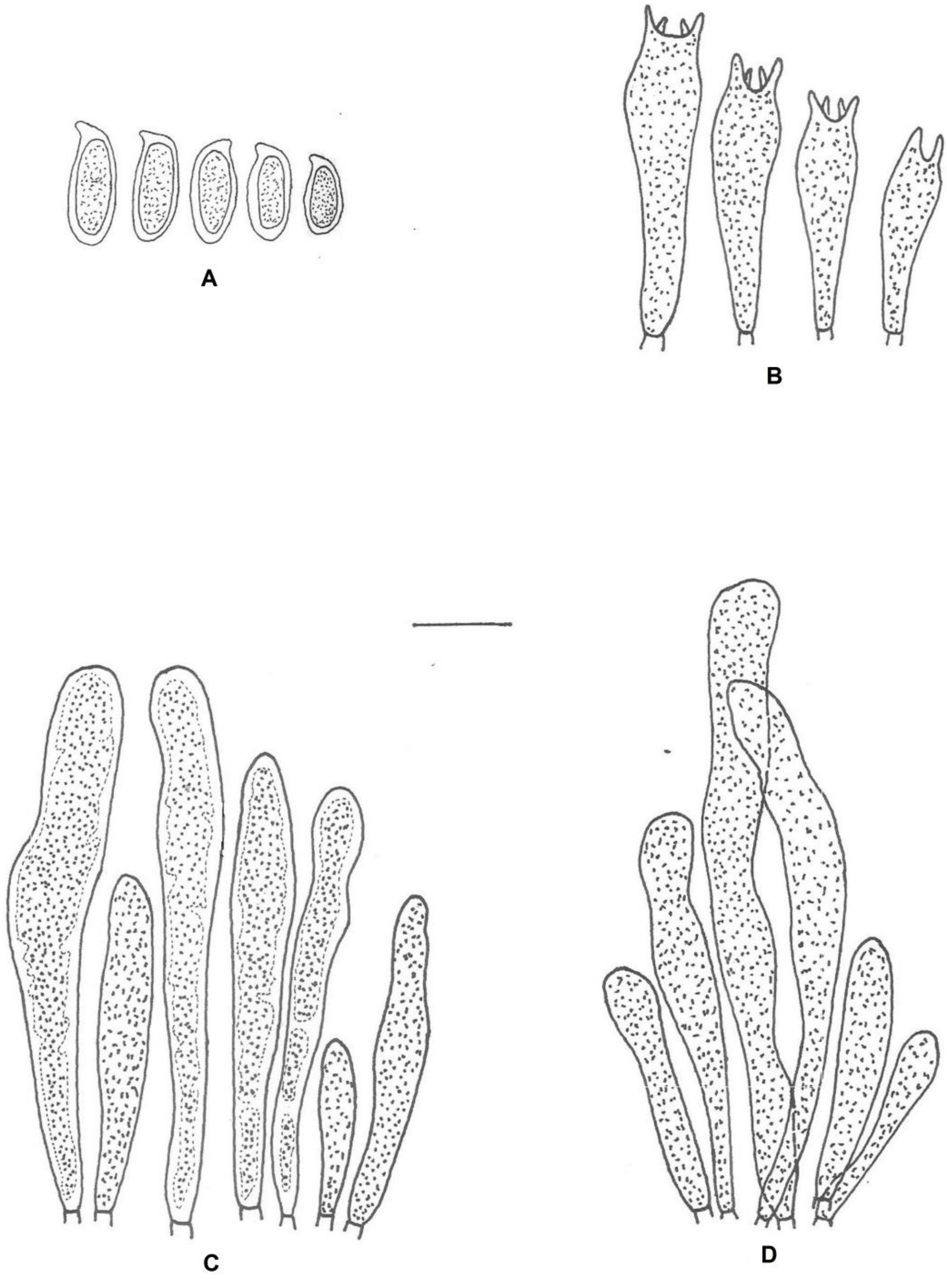


Fig. 4.4 *Suillus indicus*, microscopic line drawings: **A.** Basidiospores; **B.** Basidia; **C.** Pleurocystidia; **D.** Cheilocystidia. Scale bar: A–D = 10 μm

CHEMICAL COLOR REACTIONS: Pileal flesh: 2.5% KOH— pink then to bluish gray, 10% $FeSO_4$— olive gray, 14% ammonia— pinkish red then to bluish gray, conc.

HNO_3— no color reaction. Pileus cuticle: 2.5% KOH— greenish black, 10% $FeSO_4$— blue black, 14% ammonia— dark green to blue black, conc. HNO_3— no color reaction. HABITAT: Solitary on humicolous soil under *C. deodara* trees in mixed forest of *P. wallichiana* and *C. deodara*.

OTHER SPECIMEN EXAMINED: INDIA. Himachal Pradesh: Shimla, Kandyali, 2450 m, 29th July 2011, *B. Verma* (PUN 6578).

3. *Suillus himalayensis* sp. nov.

Suillus himalayensis B. Verma & M. S. Reddy, **sp. nov.** (Figs. 4.5&4.6)

MycoBank no.: MB 805647.

ETYMOLOGY: The specific epithet "*himalayensis*" refers to the new *Suillus* species being reported from the Himalayan region of India.

DIAGNOSIS: The fungus resembles *Suillus americanus* (Peck) Snell, but differs morphologically by the yellow white to pale yellow pileus with grayish greenish tinge, brown colored appressed fibrillose scales, white colored pileal veil, and the pileal context changing to grayish green and tubes to olive brown on bruising. Microscopically, more cylindrical spores, larger basidia, and less broaden cystidial elements distinguish it from *Suillus americanus*.

TYPE: INDIA. Himachal Pradesh: Mandi, Barot, Lachkandi, 1829 m, 7th August 2011, *B. Verma* (PUN 5535).

DETAILED DESCRIPTION:

Macrocharacters: Basidiocarps 3.0–11.5 cm in height. Pileus 3.5–8.0 cm broad, convex when young, flattened with age; margin irregular and slightly upturned; pileal veil white (1A1), cottony, collapsing with age and finally evanescent; pileus surface moist to visc-

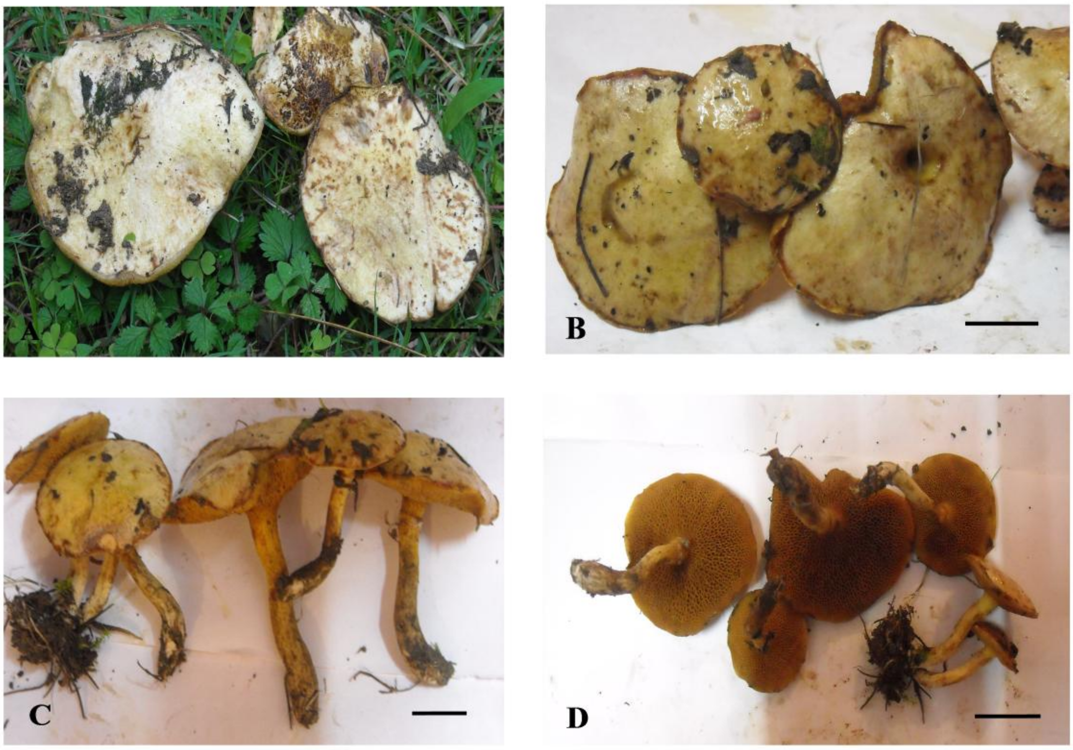


Fig. 4.5 *Suillus himalayensis* basidiocarps: **A&B.** Basidiocarps showing light greenish tinge and brownish squamules over the pileal surface; **C&D.** Basidiocarps showing tubes, stipe, and veil remnants attached to the stipe. Scale bars: A–D=2 cm

id, yellowish white (1A2) to pale yellow (1A3) with grayish green (1C3–1C5) tinge, cuticle fully peeling, umbo absent; scales brown colored appressed fibrillose throughout the surface, more prominent on the margins. Pileus context 5–15 mm thick, pale yellow (1A3), turning grayish green on bruising; odor not distinctive and taste mild. Tubes 5–7 mm deep, decurrent, radial, crowded, fully peeling, pale yellow (2A3) when young, grayish yellow (2C4–2C5) with age, slowly staining (3–5 min) to olive brown on bruising; pore mouths angular, large, 1–2 mm broad. Stipe central to excentric, 2.8–8.5 cm long, 4–12 mm thick, tubular and equal in diameter, covered with brown black glandular dots throughout, color yellow (2A5) at top, pale yellow (2A3) in middle and white (1A1) at base, grayish yellow to olive brown when handled; annulus lacking, veil remnants attached to the stipe in rare specimens; mycelium at base white. Stipe context solid, pale yellow (1A3), and changing grayish green when bruised.

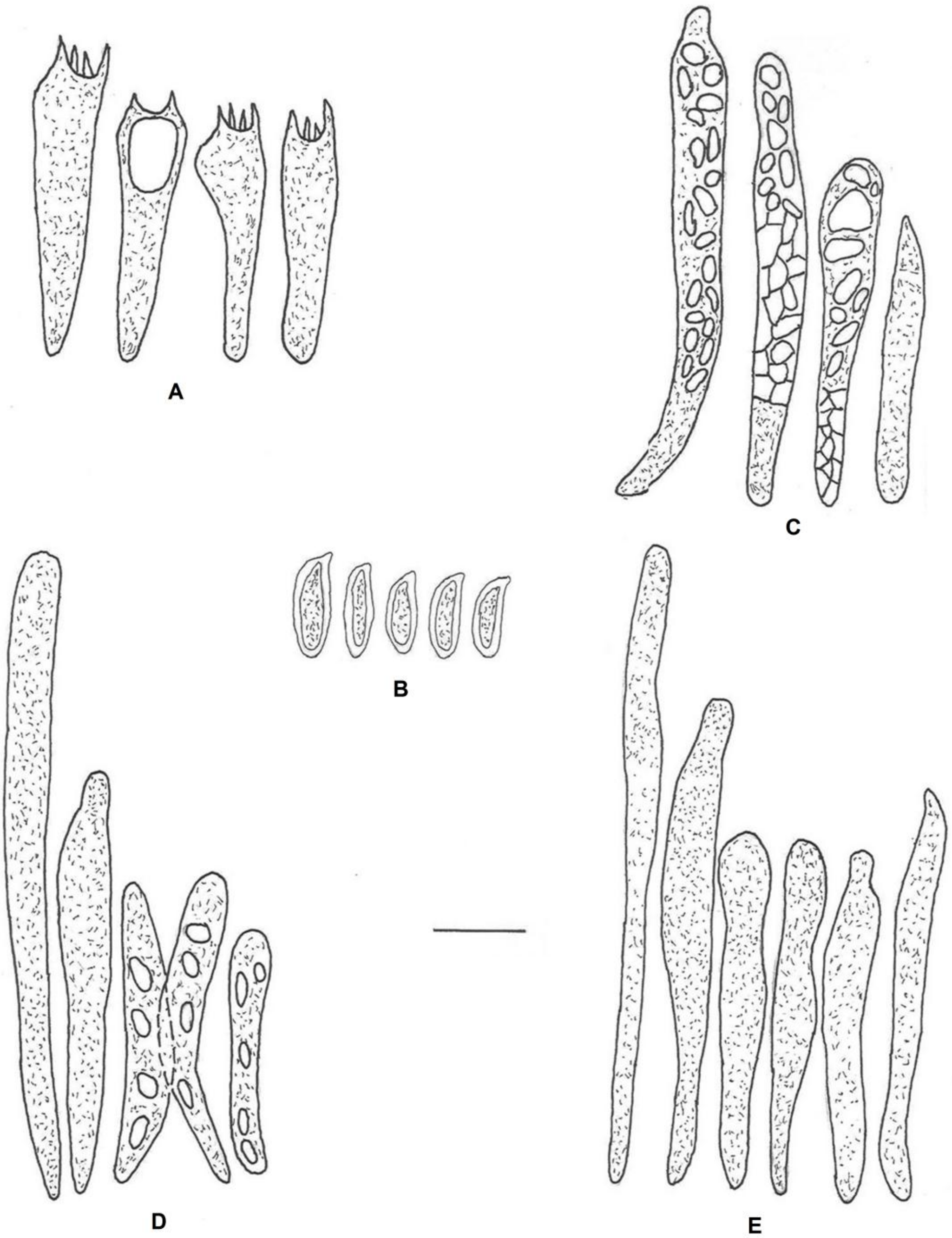


Fig. 4.6 *Suillus himalayensis*, microscopic line drawings: **A.** Basidia; **B.** Basidiospores; **C.** Pleurocystidia; **D.** Cheilocystidia; **E.** Caulocystidia. Scale bar: A–E=10 μm

Microcharacters: Spore deposit light brown (6D8), spores 9–12 × 3.0–3.8 µm (Q=3.0), cylindrical, hyaline to brownish in KOH, yellowish in Melzer's, smooth, and granular content inside. Basidia 22–30 × 6–8 µm, clavate, granular, 4 spored, occasionally 2 spored, hyaline to yellowish in KOH, and yellowish in Melzer's; sterigmata 1.5–4.6 µm high. Pleurocystidia 30–55 × 4.6–6.2 µm, cylindrical to subclavate with acute, capitate to blunt tips, granular mostly with brown coagulated contents in cystidia, in fascicles or scattered, hyaline to slightly brownish in KOH, and yellowish brown in Melzer's. Cheilocystidia 26–70 × 3.8–6.2 µm, abundant, mostly in fascicles, and almost similar to pleurocystidia. Caulocystidia at apex 38–70 × 4.6–6.2 µm, cylindrical to subclavate with acute, capitate and blunt tips, granular without any brown contents, and absent at base. Trama divergent and clamp connections absent.

CHEMICAL COLOR REACTIONS: Pileal flesh: 2.5% KOH— reddish, 10% $FeSO_4$— olive, 14% ammonia— reddish brown then to black, conc. HNO_3— no color reaction. Pileus cuticle: 2.5% KOH— light brown then to blue black, 10% $FeSO_4$— blackish brown, 14% ammonia— brown then to black brown, conc. HNO_3— no color reaction.

HABITAT: Solitary to scattered on humicolous soil under young to moderate aged *Pinus wallichiana* trees.

OTHER SPECIMEN EXAMINED: INDIA. Himachal Pradesh: Mandi, Barot, Tikkan, 1829 m, 28th July 2012, *B. Verma* (PUN 5537).

4. *Suillus granulatus* (L.) Roussel (Figs. 4.7&4.8)

DETAILED DESCRIPTION:

Macrocharacters: Basidiocarps 3.0–10.0 cm in height. Pileus 1.5–9.5 cm broad, convex becoming broadly convex with age; margin regular to irregular; pileal veil absent; pileus

Fig. 4.7 *Suillus granulatus* (basidiocarps): **A.** Scattered growth pattern of sporocarps; **B,C&D.** Sporocarps showing yellowish brown to light brown to cinnamon brown pileus with glabrous, streaked or cracked pileal surface; **E&F.** Young sporocarps with grayish orange or pallid pileal surface; **G&H.** Sporocarps showing yellowish white to pale yellow tubes and white colored stipe becoming pale yellow at top with maturity; **I.** Sporocarps showing yellow tubes and pinkish tan to vinaceous brown glandular dots or smears throughout the stipe surface. Scale bars: A=0.5 cm; B–I=2 cm

surface dry to moist, viscid only when wet, yellowish brown (5D8) to light brown (6D4–6D5), to cinnamon brown (6D6), glabrous or streaked or spotted with brownish ground color, young specimens pallid or grayish orange (5B3–5B4), cuticle fully peeling, umbo and scales absent. Pileus context yellowish white (1A2) to pale yellow (1A3), not staining when bruised up to 25 mm thick, watery greenish immediately above the tubes; odor not distinctive and taste mild. Tubes 2–7 mm deep, adnate to sub-decurrent, close, fully peeling, yellowish white (1A2) to pale yellow (1A3) when young, soon yellow (2A6–2A7) becoming olive yellow (2C8–2D8) with age, unchanging on bruising, spotted light brown to brown with age; pore mouths angular, small, 1–3 per mm, often with cloudy droplets when young. Stipe central, 2.5–7.0 cm long, 4–12 mm thick (upto 15 mm at top), tubular and equal in diameter, tapering at base in few specimens, covered with pinkish tan to vinaceous brown glandular dots or smears throughout, color white (1A1) throughout in young specimens, becoming pale yellow (1A3) at top with maturity; annulus absent; mycelium at base white. Stipe context soft, unchanging, white at first and then becoming pale yellow (1A3) in age. *Microcharacters*: Spore deposit light brown (dull cinnamon, 6D8), spores 7.0–9.5 × 2.3–3.8 µm (Q=2.8), cylindrical in shape, yellowish in KOH and Melzer's reagent, smooth. Basidia 16.0–24.0(30) × 4.6–6.2 µm, sub-cylindrical to clavate, 4 spored, hyaline in KOH and yellowish in Melzer's reagent; sterigmata 2.3–3.1 µm high. Pleurocystidia 38.0–62.0 × 5.0–8.0 µm, clavate, in fascicles incrusted with brown particles at base, hyaline with brown coagulated contents in KOH, pale yellowish with brownish contents in Melzer' reagent. Cheilocystidia 28.0–58.0 × 6.0–9.2 µm, mostly in fascicles, clavate, hyaline in KOH, pale yellowish in Melzer's reagent, uniformly distributed brown content inside. Caulocystidia 37.0–56.0 × 6.0–9.0 µm, clavate or cylindrical, fasciculate, content hyaline with brown coagulated material, base incrusted with brown particles. Trama gelatinous and divergent. Clamp connections absent.

CHEMICAL COLOR REACTIONS: Pileal flesh: 2.5% KOH— reddish, 10% $FeSO_4$— grayish green, 14% ammonia— reddish then to reddish brown, conc. HNO_3— no color reaction. Pileus cuticle: 2.5% KOH— light brown then to blue black, 10% $FeSO_4$—

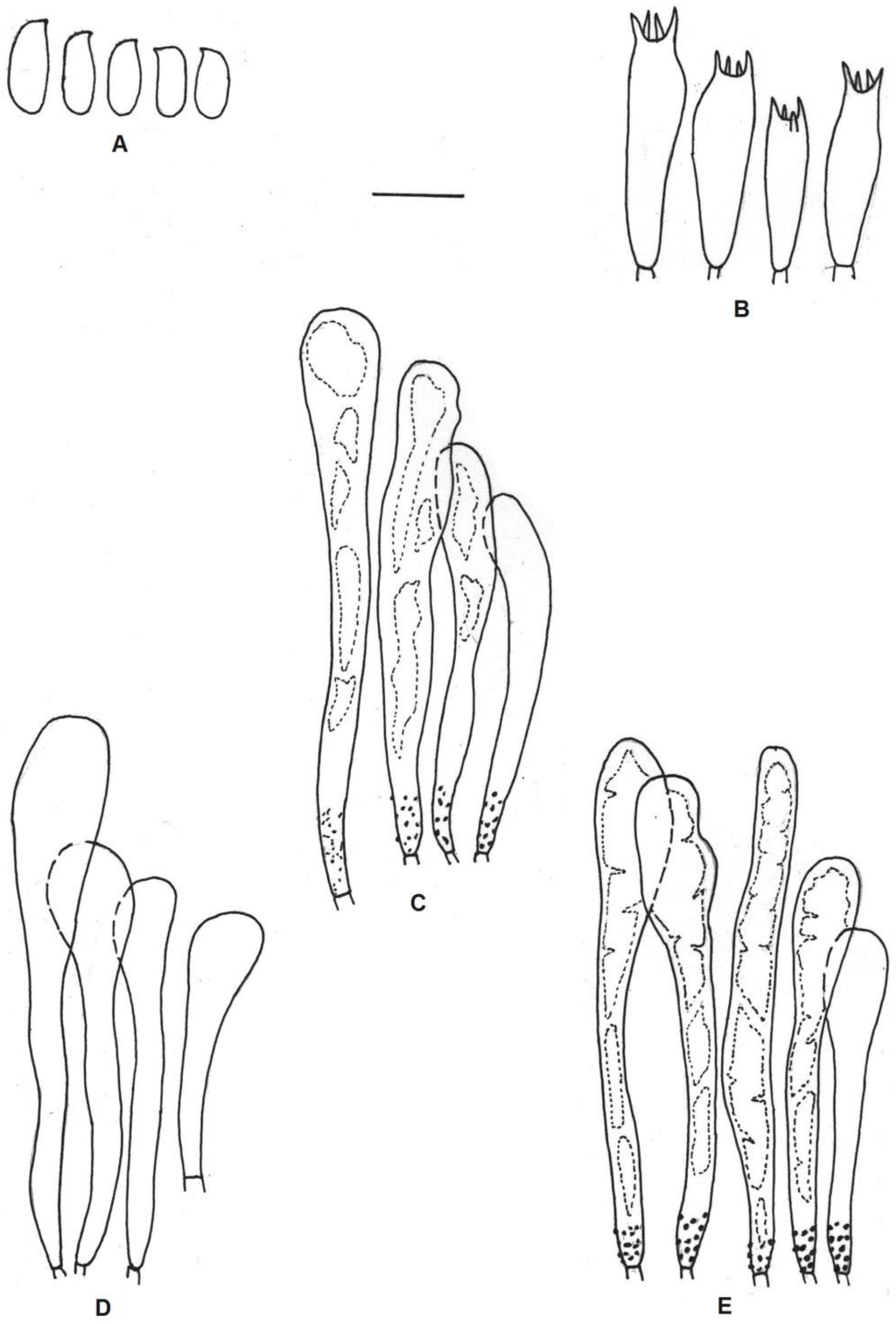


Fig. 4.8 *Suillus granulatus*, microscopic line drawings: **A.** Basidiospores; **B.** Basidia; **C.** Pleurocystidia; **D.** Cheilocystidia; **E.** Caulocystidia. Scale bar: A–E=10 μm

olive gray, 14% ammonia— brown then to black brown, conc. HNO_3— no color reaction.

HABITAT AND DISTRIBUTION: Solitary, scattered to gregarious on humicolous soil associated mostly with *Pinus wallichiana* trees, but a few specimens (SHP21 and SHP34) were also recorded with *C. deodara* trees. *Suillus granulatus* is second most abundant *Suillus* species after *S. sibiricus* found in the northwestern Himalayas. Its distribution ranges from 1500 m to 3000 m.

SPECIMENS EXAMINED: INDIA. Jammu and Kashmir: Kulgam, Aharbal, 2266 m, 5th August 2009, *B. Verma* (SJK2); Anantnag, Kukernag, 2000 m, 21st July 2010, *B. Verma* (SJK13). Himachal Pradesh: Shimla, Kumarsain, Oddi, 1850 m, 26th July 2011, *B. Verma* (SHP4); Kullu, Anni, Dagsari, 2350 m, 25th July 2012, *B. Verma* (SHP21); Chamba, Tissa, 1870 m, 5th August 2012, *B. Verma* (SHP28); Kinnaur, Kalpa, 2960 m, 18th July 2013, *B. Verma* (SHP34). Uttarakhand: Uttarkashi, Barkot, 1524 m, 29th July 2010, *B. Verma* (SUK2); Chamoli, Naagnath, 1615 m, 2nd August 2010, *B. Verma* (SUK5); Chamoli, Gwaldam, 1708 m, 6th August 2010, *B. Verma* (SUK7); Tehri Garhwal, Dhanaulti, 2286 m, 22nd August 2013, *B. Verma* (SUK27).

5. *Suillus sibiricus* (Singer) Singer (Figs. 4.9a,b&4.10)

DETAILED DESCRIPTION:

Macrocharacters: Basidiocarps 4.0–9.0 cm in height. Pileus 3.5–10.0 cm broad, convex, expanding to plane with slight umbo in few specimens, flattened with age; margin regular and incurved in young specimenns and upturned with age; appendiculate with white (1A1) cottony pileal veil, collapsing with age and finally evanescent; pileus surface viscid to glutinous, white (1A1), yellowish white to light yellow (1A2–1A5),

Fig. 4.9a *Suillus sibiricus* (basidiocarps): **A,B&C.** Sporocarps showing yellowish white pileal surface with patches of reddish brown squamules over the pileus; **D&E.** Sporocarps with glabrous white pileal surface; **F,G,H&I.** Sporocarps with glabrous, yellowish white to light yellow pileal surface. Scale bars: A–I=2 cm

brownish yellow (5C7–5C8) or dingy olive yellow in age, cuticle fully peeling, umbo absent; scales either absent or if present then streaked to appressed fibrillose, ranging from reddish brown through light brown to dark brown in color, distributed throughout the pileal surface or towards periphery. Pileus context pale yellow (1A3/2A3/3A3), cinnamon brown on bruising, mostly upto 12 mm thick (15 mm in few specimens); odor not distinctive and taste mild. Tubes 5–10(15) mm deep, decurrent, radial, crowded, fully peeling, pale yellow to yellow (2A3/3A3–2A7/3A7) with age, staining cinnamon

Fig. 4.9b *Suillus sibiricus* (basidiocarps): **J,K&L.** Sporocarps showing brownish yellow pileal surface with reddish brown through light brown to dark brown appressed fibrillose squamules all over the pileus; **M&N.** Sporocarps showing brownish yellow pileal surface with streaks of scales over the pileus **O&P.** Sporocarps showing pale yellow tubes (turning cinnamon brown on bruising) and stipe; **Q&R.** Sporocarps showing yellow tubes, pale yellow stipe (becoming vinaceous to reddish at base) and veil remnants on the stipe. Scale bars: J–R=2 cm

brown on bruising; pore mouths angular, large, 0.5–2.0 mm broad. Stipe central, 3.0–8.0 cm long, 6–12 mm thick (rarely 15mm), tubular and equal in diameter, covered throughout with glandular dots darkening in mature specimens, concolorous with tubes, base whitish (1A1) base soon becoming vinaceous to reddish, staining dull brown when handled; prominent annulus present in few specimens, mostly veil remnants attached to the stipe; mycelium at base white. Stipe context solid, pale yellow (1A3/2A3/3A3), and changing cinnamon brown when bruised.

Microcharacters: Spore deposit olive brown to light brown (6D6–6D8). Spores 7.2–10.8 × 3.0–4.6 µm, oblong to narrowly cylindric in shape (Q= 2.3), yellowish in KOH, dark yellowish in Melzer's reagent, smooth. Basidia 4 spored, 18.5–28.6 × 4.3–7.2 µm, clavate, hyaline in KOH, yellowish in Melzer's; sterigmata 2.3–3.8 µm high.. Pleurocystidia in bundles, individual cystidia 30.0–70.0 × 4.0–9.0 µm, cylindric to narrowly clavate with blunt rounded ends, often crooked and contains brownish contents inside. Cheilocystidia similar to pleurocystidia. Caulocystidia 35.0–80.0(95.0) × 4.0–8.0, with brown coagulated contents inside, rest similar to pleurocystidia. Tube trama is gelatinous made up of divergent hyaline hyphae. Clamp connections absent.

CHEMICAL COLOR REACTIONS: Pileal flesh: 2.5% KOH— reddish, 10% $FeSO_4$— yellowish green, 14% ammonia— reddish brown then to black, conc. HNO_3— no color reaction. Pileus cuticle: 2.5% KOH— light brown then to blue black, 10% $FeSO_4$— blackish brown, 14% ammonia— brown then to black brown, conc. HNO_3— no color reaction.

HABITAT AND DISTRIBUTION: Solitary to scattered and gregarious under coniferous trees of *Pinus wallichiana*, *Cedrus deodara* and *Picea smithiana*. Associated mainly with *Pinus wallichiana*, very few records (SHP37 and SUK26) found with *Cedrus deodara* and only one specimen (SUK20) found with *Picea smithiana*. *Suillus sibiricus* sporocarps are distributed throughout the northwestern Himalayas ranging from J.&K. to U.K. and were noticed to be frequently occurring and most dominant *Suillus* species in the northwestern Himalayas.

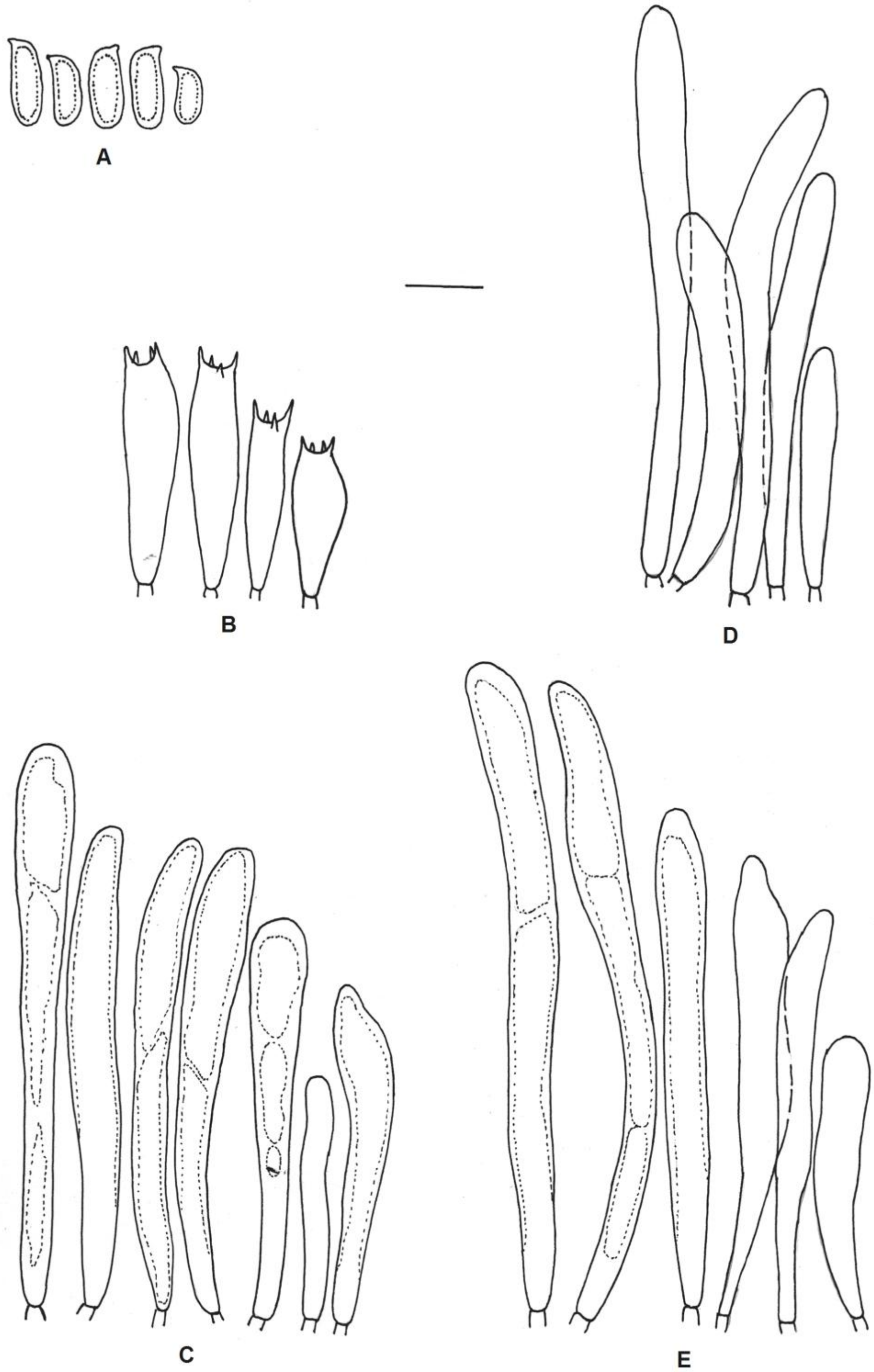


Fig. 4.10 *Suillus sibiricus*, microscopic line drawings: **A.** Basidiospores; **B.** Basidia; **C.** Pleurocystidia; **D.** Cheilocystidia; **E.** Caulocystidia. Scale bar: A–E=10 μm

SPECIMENS EXAMINED: INDIA. Jammu and Kashmir: Ganderbal, Mamar, 1916 m, 2nd August 2009, *B. Verma* (SJK1); Kulgam, Aharbal, 2266 m, 5th August 2009, *B. Verma* (SJK3); Anantnag, Pahalgam, 2740 m, 20th July 2010, *B. Verma* (SJK12). Himachal Pradesh: Shimla, Kharapathar, 2673 m, 25th July 2010, *B. Verma* (SHP2); Shimla, Rohru, Shallan, 1900 m, 26th July 2011, *B. Verma* (SHP5); Mandi, Karsog, Seri, 2065 m, 6th August 2011, *B. Verma* (SHP12); Kullu, Banjar, Jibhi, 2300 m, 25th July 2012, *B. Verma* (SHP22); Kullu, Banogi, 1340 m, 26th July 2012, *B. Verma* (SHP23); Chamba, Bharmour, 2133 m, 3rd August 2012, *B. Verma* (SHP30); Sirmaur, Shillai, 1900 m, 28th July 2013, *B. Verma* (SHP35); Sirmaur, Haripurdhar, 2500 m, 31st July 2013, *B. Verma* (SHP37); Sirmaur, Tarna, 1969 m, 2nd August 2013, *B. Verma* (SHP38). Uttarakhand: Chamoli, Joshimath, 1890 m, 4th August 2010, *B. Verma* (SUK6); Nainital, Mukteshwar, 2290 m, 21st August 2011, *B. Verma* (SUK12); Uttarkashi, Janki Chatti, 2650 m, 11th August 2012, *B. Verma* (SUK20); Uttarkashi, Harsil, 2620 m, 13th August 2012, *B. Verma* (SUK23); Tehri Garhwal, Chandrabadni, 2277 m, 18th August 2013, *B. Verma* (SUK26).

6. *Suillus flavidus* (Fr.) Singer (Figs. 4.11&4.12)

DETAILED DESCRIPTION:

Macrocharacters: Basidiocarps 5.0–12.0 cm in height. Pileus 3.0–8.0 cm broad, convex, expanding to plane with slight umbo in few specimens at maturity, flattened with age; margin regular and slightly upturned with age; appendiculate with white (1A1) cottony pileal veil; pileus surface viscid to glutinous, glabrous with patches of gluten when dried, yellow (2A4–2A6) to yellowish brown (5C7–5C8) in age, cuticle fully peeling. Pileus context pale yellow (2A3), soft, browning when cut, up to 10 mm thick; odor not distinctive and taste mild. Tubes 3–9 mm deep, adnate to sub-decurrent,

Fig. 4.11 *Suillus flavidus* (basidiocarps): **A.** Scattered growth pattern of sporocarps; **B.** Sporocarp showing yellow and glabrous pileus with patches of gluten; **C.** Sporocarp showing angular to irregular pore mouths and a thick annulus; **D.** Pinkish red to reddish stipe of mature specimens. **E&F.** Sporocarps showing tubes, annulus and stipe. Scale bars: A–F=2 cm

radial, crowded, fully peeling, yellow (3A6–3A7), slowly staining cinnamon brown to pinkish brown on bruising; pore mouths angular to irregular, large, 1–2 mm broad. Stipe central, solid, 3–7 cm long, 5–12 mm thick, tubular and equal in diameter, color pale yellow (2A3) to yellow (2A5), soon becoming pinkish red or reddish with age, covered with pallid to pale yellowish glandular dots, darkening when handled or with age; whitish (1A1) thick band of annulus present, staining brownish on handling; mycelium at base white. Stipe context solid, pale yellow (2A3), and changing cinnamon brown when bruised.

Microcharacters: Spore print light brown (6D8), spores cylindrical in shape, smooth, 7.5–10.8 × 3.0–3.6 µm (Q=2.7) in size, yellowish in KOH and yellowish brown in Melzer's. Basidia 18.0–28.0 × 5.0–8.0 µm, clavate, hyaline in KOH, yellowish brown in Meltzer's, 1–4 spored; sterigmata 1.5–3.1 µm high. Pleurocystidia 38.0–70.0 × 5.0–8.0 µm, scattered to fasciculate with brown incrusting particles around the base, clavate to cylindrical, content hyaline to yellowish brown. Cheilocystidia 38.0–62.0 × 6.0–8.0 µm, abundant with uniform brown content, shape similar to pleurocystidia. Caulocystidia 35.0–75.0 × 6.0–8.0 µm, in fascicles, surrounded by brown incrusting particles at the base of cystidia. Tube trama gelatinous and divergent. Clamp connections absent.

CHEMICAL COLOR REACTIONS: Pileal flesh: 2.5% KOH— reddish brown, 10% $FeSO_4$— yellowish green, 14% ammonia— reddish brown, conc. HNO_3— no color reaction. Pileus cuticle: 2.5% KOH— light brown then to blue black, 10% $FeSO_4$— blackish brown, 14% ammonia— brown then to brownish black, conc. HNO_3— no color reaction.

HABITAT AND DISTRIBUTION: Found scattered in pure forests of *P. wallichiana* on humicolous soil.

SPECIMENS EXAMINED: INDIA. Himachal Pradesh: Shimla, Baghi, 2685 m, 25th July 2010, *B. Verma* (SHP1); Mandi, Karsog, Chindi, 1825 m, 6th August 2011, *B. Verma* (SHP14).

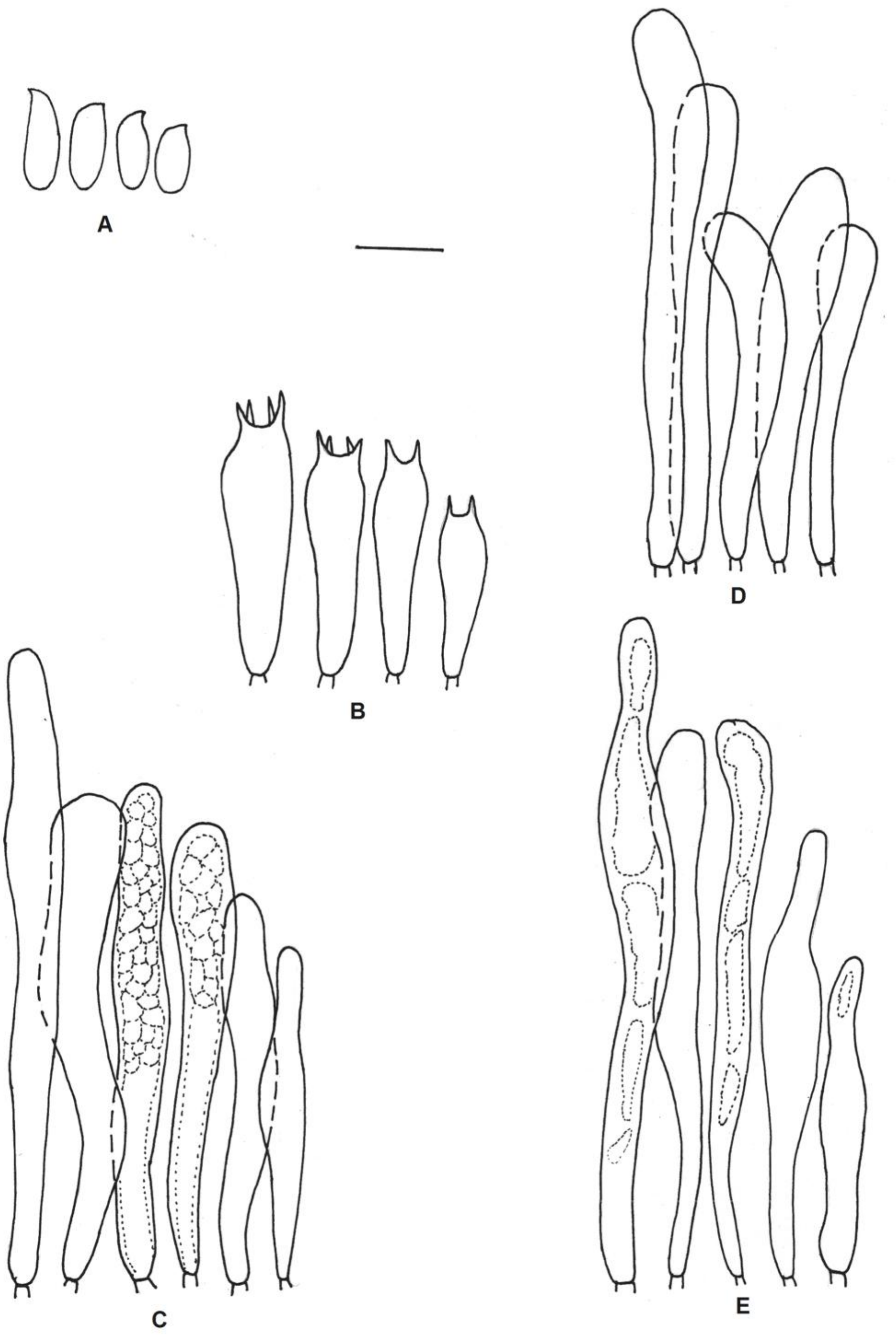


Fig. 4.12 *Suillus flavidus*, microscopic line drawings: **A.** Basidiospores; **B.** Basidia; **C.** Pleurocystidia; **D.** Cheilocystidia; **E.** Caulocystidia. Scale bar: A–E=10 μm

7. *Suillus placidus* (Bonord.) Singer (Figs. 4.13&4.14)

DETAILED DESCRIPTION:

Macrocharacters: Basidiocarps 4.0–12.0 cm in height. Pileus 3.0–8.5 cm broad, broadly convex becoming plane with age; margin regular, inrolled when young; pileal veil absent; pileus surface viscid to glutinous, glabrous, white (1A1) when young becoming pale yellow (1A2–1A3) in age, cuticle fully peeling, umbo and scales absent. Pileus context white (1A1) to yellowish white (1A2), pale yellow (1A3) adjacent to tubes, slowly turning vinaceous on bruising, upto 14 mm thick; odor not distinctive and taste mild. Tubes 4–10 mm deep, adnate to decurrent, radial, crowded, fully peeling, pale yellow (1A3) at maturity, unchanging on bruising; pore mouths angular, 0.5–2.0 mm broad, often with pinkish droplets of exudates and with pinkish glandular dots. Stipe central, 4–12 cm long, 3–10 mm thick, tubular and equal in diameter, covered with vinaceous brown glandular dots or smears throughout, color white (1A1), pale yellow (1A3) at top in mature specimens; veil or annulus absent during all stages of development; mycelium at base white. Stipe context soft, white, becoming pale yellow with age, slowly turning vinaceous at base when bruised.

Microcharacters: Spore deposit dull cinnamon (6D6), spores 7.0–9.5 × 2.8–3.5 μm (Q=2.24) in size, oblong to narrowly cylindrical in shape, smooth, greenish hyaline in KOH, pale yellow in Melzer's reagent. Basidia 18.0–28.0 × 5.5–8.0 μm, clavate, 4 spored, hyaline in KOH and yellowish in Melzer's reagent; sterigmata 1.5–3.1 μm high. Pleurocystidia abundant, 30.0–70.0 × 5–9 μm, sub-cylindrical to clavate , scattered, but typically in fascicles, hyaline in KOH, pale yellowish in Melzer' reagent. Cheilocystidia 30.0–85.0 × 5.0–14.0 μm, in fascicles, clavate to cylindrical with inflated ends, brownish in KOH and light brown in Melzer's reagent. Caulocystidia 30.0–70.0 × 6.0–

18.0 µm, found mostly in bundles with capitate ends, brown in KOH. Brown incrusting particles found at the base of each cystidia. Trama gelatinous and divergent. Clamp connections absent.

Fig. 4.13 *Suillus placidus* (basidiocarps): **A,B&C.** Sporocarps with white to pale yellow pileal surface; **D.** Sporocarps showing pale yellow and crowded tubes; **E&F** Sporocarps with light brown to brown spotted tubes and vinaceous brown glandular dots on the stipe. Scale bars: A–F=2 cm

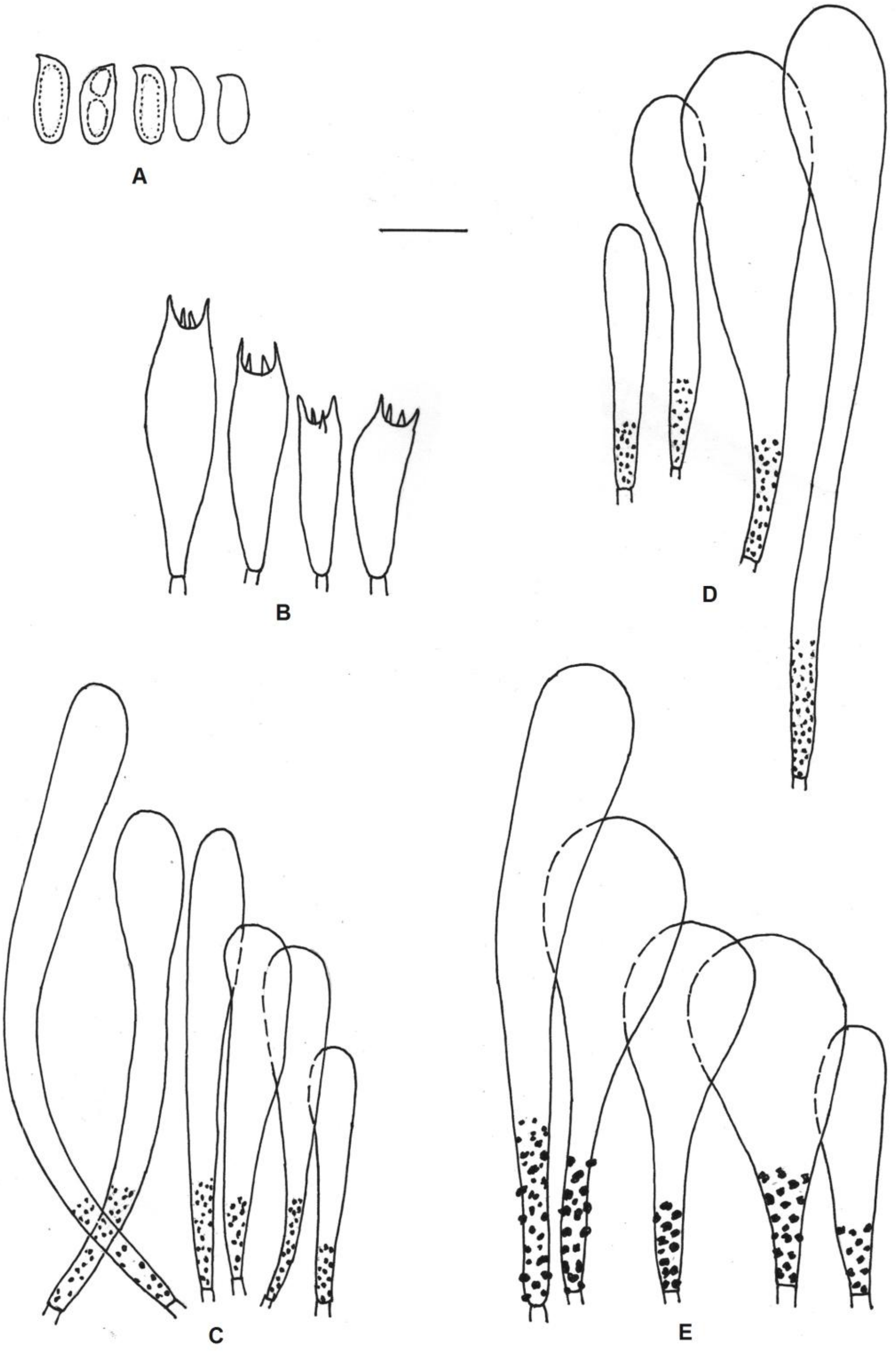


Fig. 4.14 *Suillus placidus*, microscopic line drawings: **A.** Basidiospores; **B.** Basidia; **C.** Pleurocystidia; **D.** Cheilocystidia; **E.** Caulocystidia. Scale bar: A–E=10 μm

CHEMICAL COLOR REACTIONS: Pileal flesh: 2.5% KOH— pink then to brownish lavender, 10% $FeSO_4$— gray or olive brown, 14% ammonia— pink becoming blue gray, conc. HNO_3— no color reaction. Pileus cuticle: 2.5% KOH— pink becoming brownish black, 10% $FeSO_4$— brown to blackish brown,14% ammonia— pink becoming red or finally black, conc. HNO_3— no color reaction.

HABITAT AND DISTRIBUTION: Solitary to scattered or gregarious on humicolous soil under *Pinus wallichiana* trees.

SPECIMENS EXAMINED: INDIA. Himachal Pradesh: Shimla, Theog, 1965 m, 10th July 2013, *B. Verma* (SHP32). Uttarakhand: Tehri Garhwal, Tehri, 1750 m, 16th August 2012, *B. Verma* (SUK24).

Field Key to the northwestern Himalayan species of *Suillus* investigated

1 Glandular dots absent on the stipe surface ……………………………..….. ***S. indicus***

1*Glandular dots present on the stipe surface …………………………………....… **2**

2 A well developed veil or false veil found in young specimens either leaving an annular zone or true annulus on the stipe, or otherwise adhered to the pileal margin at maturity …………………………………………………………………… **3**

2. Pileal veil and annulus absent during all stages of the development ………………... **5**

3 Pileus yellow white to pale yellow with light greenish tinge; cystidial elements less broaden (3.8–6.2 µm) …..…………………………………….…. ***S. himalayensis***

3*Pileus not as above, cystidial elements comparatively broader .....………..………… **4**

4 Pileus yellow to yellowish brown, usually umbonate, glabrous; stipe with distinct annulus ..………………………………………………………….……... ***S. flavidus***

4*Pileus white, yellowish white through light yellow to brownish yellow or dingy olive yellow in age, usually plane, rarely obtusely umbonate, mostly covered with appressed fibrillose scales; either prominent annulus present or otherwise veil remnants attached to the stipe ....………….. ***S. sibiricus***

5. Associated exclusively with *Pinus roxburghii* …………..…………..... ***S. triacicularis***

5*Associated with other conifers ……………………………………………………. **6**

6 Pileus white when young, slowly becoming pale yellow with age; pileal context not watery greenish above the tubes; associated only with *Pinus wallichiana* ………………………..…………...................................………….. ***S. placidus***

6*Pileus pallid or grayish orange when young, becoming yellowish brown to light brown or cinnamon brown with age; pileal context watery greenish immediately above the tubes; associated either with *Pinus wallichiana* or with *Cedrus deodara* ……....……..…... ***S. granulatus***

4.1.4 Isolation of pure cultures

All of the *Suillus* isolates were isolated on Malt Extract (ME) agar (2% w/v) media. Although a few cultures were obtained also on Modified Melin-Norkrans (MMN) agar media (Marx 1969), but the growth rate was comparatively low or otherwise poor. PDA (Potato dextrose agar) media was not found satisfactory for isolating *Suillus* culture as we didn’t get any culture isolated on PDA agar. Total of eight cultures were isolated from fresh basidiocarps (Table 4.2). The corresponding collection number and PUN number of basidiocarps from which cultures were isolated are also mentioned in Table 4.2. The cultures were designated from “SNW01–SNW08” (SNW stands for ‘*Suillus* species from north western Himalayas’) and have been submitted to Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India under the accession number shown in Table 4.2.

Table 4.2 Different *Suillus* cultures isolated from the northwestern Himalayas, India

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

4.1.5 Identification of *Suillus* species based on Molecular taxonomy

Initially, all the *Suillus* specimens collected were identified and classified by phenotypic and microscopic examination of basidiocarps. Based on species categorization by classical taxonomic methods, a few specimens for each species were selected for molecular analysis. In total, 21 basidiocarps of different species were selected to confirm species identification by molecular methods and to study the inter-specific variation among the *Suillus* species. It was taken care that all the eight specimens of *Suillus* species for which cultures have been isolated (Table 4.2) were chosen for the molecular identification so as to confirm the culture purity.

4.1.5.1 ITS amplification

Genomic DNA was isolated from the 21 basidiocarps (SJK11, SHP11, SHP27, SUK03, SHP03, SHP07, SHP15, SHP26, SJK13, SHP04, SHP34, SUK07, SJK01, SJK03, SHP02, SHP05, SHP12, SUK20, SUK12, SHP01 and SHP32) and 8 cultures (SNW01, SNW02, SNW03, SNW04, SNW05, SNW06, SNW07 and SNW08) of *Suillus* species. ITS products for selected specimens and all the isolates were obtained by PCR amplification with ITS1 and ITS4 primers. The PCR products resulting from ITS amplification were analyzed by agarose gel electrophoresis using 100 bp DNA Marker (Lane M, Fig. 4.15). The result showed that the ITS locus of all the *Suillus* specimens and isolates produced a single band of approximately 700 bp (Lane 1–21, Fig. 4.15) in size. As revealed later by sequence analysis, the actual size of ITS amplicons varied from 693–702 bp.

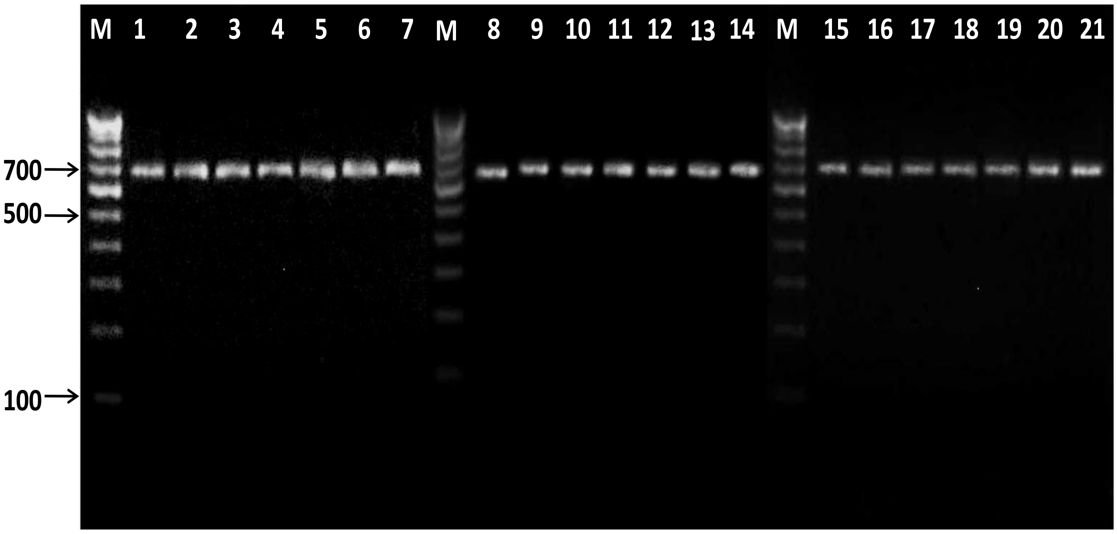


Fig. 4.15 ITS-PCR products of *Suillus* amplified with ITS1 and ITS4 primers. Lane M, 100 bp DNA marker, Lane 1-21 are amplification bands for specimens SJK11, SHP11, SHP27, SUK03, SHP03, SHP07, SHP15, SHP26, SJK13, SHP04, SHP34, SUK07, SJK01, SJK03, SHP02, SHP05, SHP12, SUK20, SUK12, SHP01 and SHP32, respectively. The figure is a composite figure made from different gels

4.1.5.2 Restriction enzyme analysis of ITS products

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 were produced using three different restriction enzymes *i.e.*, *AluI*, *HaeIII* and *MboI* (Fig 4.16). 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 60 bp were ignored during RFLP analysis. Restriction digestion with *Alu*I resulted into two, *Hae*III into four and *Mbo*I 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 (Table 4.3). Sequence analysis also revealed that these isolates belonged to five different *Suillus* species. These findings indicate that ITS-RFLP technique is an effective tool to study iner-specific variations within the genus *Suillus*.

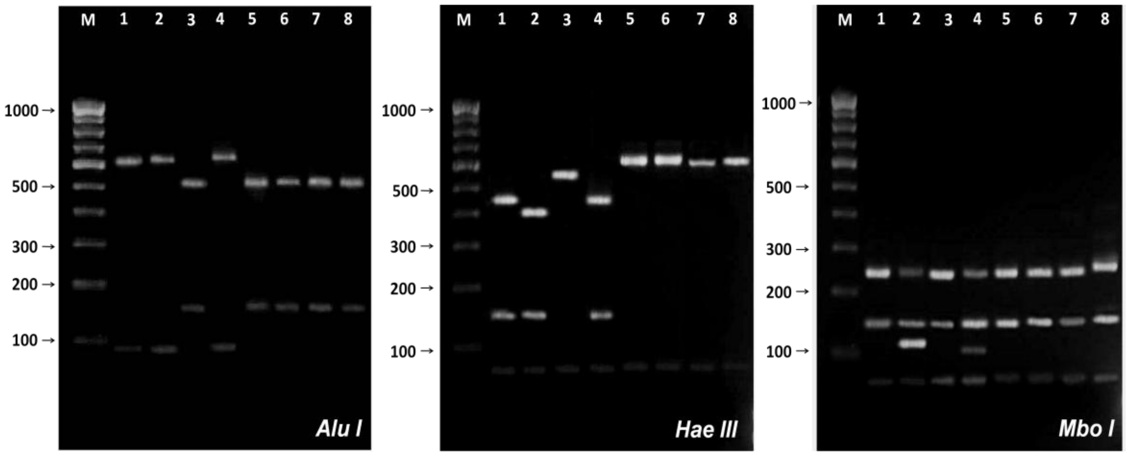


Fig. 4.16 ITS-RFLP analysis of *Suillus* isolates digested with three different restriction enzymes (*AluI*, *HaeIII*, and *MboI*). Lane M- DNA marker, Lane 1-8 are isolates of SHP27/SNW01, SHP07/SNW02, SHP26/SNW03, SJK13/SNW04, SJK01/SNW05, SHP05/SNW06, SHP12/SNW07 and SUK12/SNW08, respectively. The figure is a composite figure made from different gels

In addition, the ITS products amplified from corresponding basidiocarps of each culture were also subjected to RFLP digestions with same three restriction endonucleases. ITS-RFLP analysis of basidiocarps yielded the same restriction patterns as obtained from the corresponding cultures isolated from them. This confirmed the purity of cultures and the RFLP results were further confirmed by sequencing of the PCR amplified ITS products of each isolate.

Table 4.3 Differentiation of *Suillus* isolates into RFLP types according to the ITS-RFLP patterns produced following digestion of the ITS-PCR product with restriction enzymes

Isolate	GenBank accession No. (ITS)	ITS-RFLP pattern				ITS-RFLP taxa	*Suillus* species
		AluI	***HaeIII***	***MboI***	**Genotype**		
SNW01	KF977189	a_1	h_1	m_1	$a_1h_1m_1$	Taxon 1	*S. triacicularis*
SNW02	KJ675500	a_1	h_2	m_2	$a_1h_2m_2$	Taxon 2	*S. indicus*
SNW03	KJ472765	a_2	h_3	m_1	$a_2h_3m_1$	Taxon 3	*S. himalayensis*
SNW04	KM882912	a_1	h_1	m_3	$a_1h_1m_3$	Taxon 4	*S. granulatus*
SNW05	KM882913	a_2	h_4	m_1	$a_2h_4m_1$	Taxon 5	*S. sibiricus*
SNW06	KM882914	a_2	h_4	m_1	$a_2h_4m_1$	Taxon 5	*S. sibiricus*
SNW07	KM882919	a_2	h_4	m_1	$a_2h_4m_1$	Taxon 5	*S. sibiricus*
SNW08	KM882917	a_2	h_4	m_1	$a_2h_4m_1$	Taxon 5	*S. sibiricus*

Note: a, h and m stands for *AluI*, *HaeIII* and *MboI*, respectively; 1–4 as subscript are arbitrarily assigned numbers to represent different RFLP patterns obtained with a particular restriction enzyme; Different numbers (1–5) are assigned to represent each RFLP taxa.

4.1.5.3 Sequence analysis of ITS data

Twenty one fruit bodies of *Suillus* were subjected to ITS amplification using ITS1 and ITS4 primers and about 700 bp amplicons were observed (Fig. 4.15). ITS products were cloned into pTZ57R/T vector. The 700 bb amplified products of different clones were subjected to restriction enzymes (*AluI*, *HaeIII* and *MboI*) digestion to see the variation in the ITS region. The ITS products of selected clones were then sequenced using Applied Biosystems automatic sequencer. Sequencing reactions were performed with M13 forward and M13 reverse primers followed by their internal primers. The sequences were analyzed by multiple sequence alignment using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to check inter-specific similarities among the species. The homologies among the sequences of different *Suillus* species varied from 89.5 to 99.7%. Minimum of 89.5% similarity was found in *S. granulatus* with *S.*

placidus strains and maximum (99.1–99.7%) similarity was found between *S. himalayensis* and *S. sibiricus* strains.

Table 4.4 Examined *Suillus* species and their closest relative species inferred from ITS gene sequences of existing database

***Suillus* species**	**GenBank Accession no**	**Nearest match**	**Query coverage**	**Maximum identity**
S. triacicularis SHP27/PUN 5538	KF977189	*S. collinitus* isolate CCMA-79 (AY935517)	99%	95%
		S. collinitus isolate CCMA-46 (AY935516)	99%	95%
S. indicus SHP07/PUN 6578	KJ675500	*S. flavidus* isolate FFP962 (JQ711908)	99%	93%
		S. flavidus isolate FFP886 (JQ711885)	99%	93%
S. himalayensis SHP26/PUN 5537	KJ472765	*S. sibiricus* strain HMAS-66061 (AF166512)	98%	99%
		S. flavidus isolate FFP962 (JQ711908)	99%	98%
S. granulatus SJK13/PUN 5525	KM882912	*S. granulatus* isolate S070 (AJ272410)	98%	99%
		S. granulatus isolate AT259 (AJ272408)	98%	99%
S. sibiricus SHP05/PUN 6577	KM882914	*S. sibiricus* strain HMAS-66061 (AF166512)	98%	99%
		S. flavidus isolate FFP962 (JQ711908)	99%	98%
S. flavidus SHP01/PUN 6574	KM882920	*S. flavidus* isolate FFP962 (JQ711908)	99%	99%
		S. flavidus voucher SMI206 (FJ845439)	99%	99%
S. placidus SHP32/PUN 6584	KM882921	*S. placidus* isolate Chu37 (DQ407265)	99%	98%
		S. placidus isolate VC-1022 (L54118)	92%	99%

Search for sequence identity in the GenBank DNA database using BlastN (NCBI) (Altschul et al. 1997) revealed that the ITS region of *Suillus* species had 93% to 99% similarity (Table 4.4) with the sequences of NCBI database. The ITS sequences of *Suillus* species determined in this study were deposited in the GenBank of NCBI data library under the accession numbers mentioned in Appendix II.

The sequences of ITS region of *Suillus* species were also analyzed for restriction digestion with the help of Web cutter 2.0 (http://rna.lundberg.gu.se/cutter2/) and it was found that the restriction patterns yielded in present study were same as obtained from the webcutter (Table 4.5). The exact size of fragment was also deduced for the restriction patterns.

Table 4.5 Size of restriction fragments produced following digestion of the ITS-PCR products of *Suillus* isolates with different restriction enzymes. Exact size of fragments was deduced using web programme Web Cutter 2.0

***Suillus* Species**	**Size of fragments (bp)**		
	Alu I	*Hae III*	*Mbo I*
S. triacicularis SHP27/SNW01/PUN 5538	627, 71	465, 158, 75	238, 235, 141, 62, 22
S. indicus SHP07/SNW02/PUN 6578	636, 66	408, 157, 76, 61	243, 141, 122, 112, 62, 22
S. himalayensis SHP26/SNW03/PUN 5537	535, 158	561, 75, 57	235, 232, 142, 62, 22
S. granulatus SJK13/SNW04/PUN 5525	624, 72	461, 159, 76	233, 143, 137, 99, 62, 22
S. sibiricus SJK01/SNW05/PUN 5520	535, 158	618, 75	235, 232, 142, 62, 22
S. sibiricus SHP05/SNW06/PUN 6577	535, 158	618, 75	235, 232, 142, 62, 22
S. sibiricus SHP12/SNW07/PUN 6579	536, 158	619, 75	235, 233, 142, 62, 22
S. sibiricus SUK12/SNW08/PUN 5532	535, 158	618, 75	235, 232, 142, 62, 22

4.1.5.4 Phylogenetic inference

A phylogenetic analysis was performed to determine the evolutionary relationships. All the ITS sequences procured in the present study were compared with the available sequence database in the GenBank to retrieve the ITS sequences of the different *Suillus* species for analyzing phylogenetic relationship. A dataset was assembled for the phylogenetic analysis. The ITS dataset consisted of 77 *Suillus* taxa representing homologous ITS sequences of 28 related *Suillus* species. *Rhizopogon subcaerulescens* was included as an outgroup taxon for rooting purpose. The alignment resulted in a data matrix comprising 751 characters including gaps. The phylogenetic analysis was conducted using Bayesian inference and yielded a consensus tree as shown in Fig. 4.17. The Bayesian analysis of selected *Suillus* species clustered these *Suillus* species mainly into two different groups namely, *Suillus* group I and *Suillus* group II (Fig. 4.17).

The ITS sequences of *S. himalayensis*, *S. triacicularis,* and *S. indicus* obtained in the present study formed a well-supported monophyletic lineage when compared with ITS sequences of other related *Suillus* species. Bayesian consensus showed splits between *Suillus himalayensis* and a well-resolved clade containing *S. americanus*. Similarly, split between *S. triacicularis* and a well-resolved clade containing *S. collinitus* and *S. granulatus* was also observed. *Suillus indicus* also formed an independent clade in the group. Posterior probability percentage values support these species as separate clades in the group. As revealed morphologically, molecular analysis also identified these species as distinct *Suillus* species and new to the science. The ITS sequences of *S. sibiricus*, *S. flavidus*, *S. placidus* and *S. granulatus* are grouped with their respective homologous sequences available in the NCBI database, which further confirm their identification based upon the molecular analysis.

All the single species clades of the consensus tree received strong Bayesian posterior probabilities (BPP) support, whereas the nodes indicating relationship amongst them generally received less support. Most of the *Suillus* species formed well-supported independent clades with a few exceptions. A few pairs of *Suillus* species (*S. cothurnatus* and *S. subluteus, S. pseudobrevipes* and *S. volcanalis*, *S. glandulosipes* and *S. neoalbidipes*, *S. brevipes* and *S. weaverae*) are not distinguishable by the ITS locus.

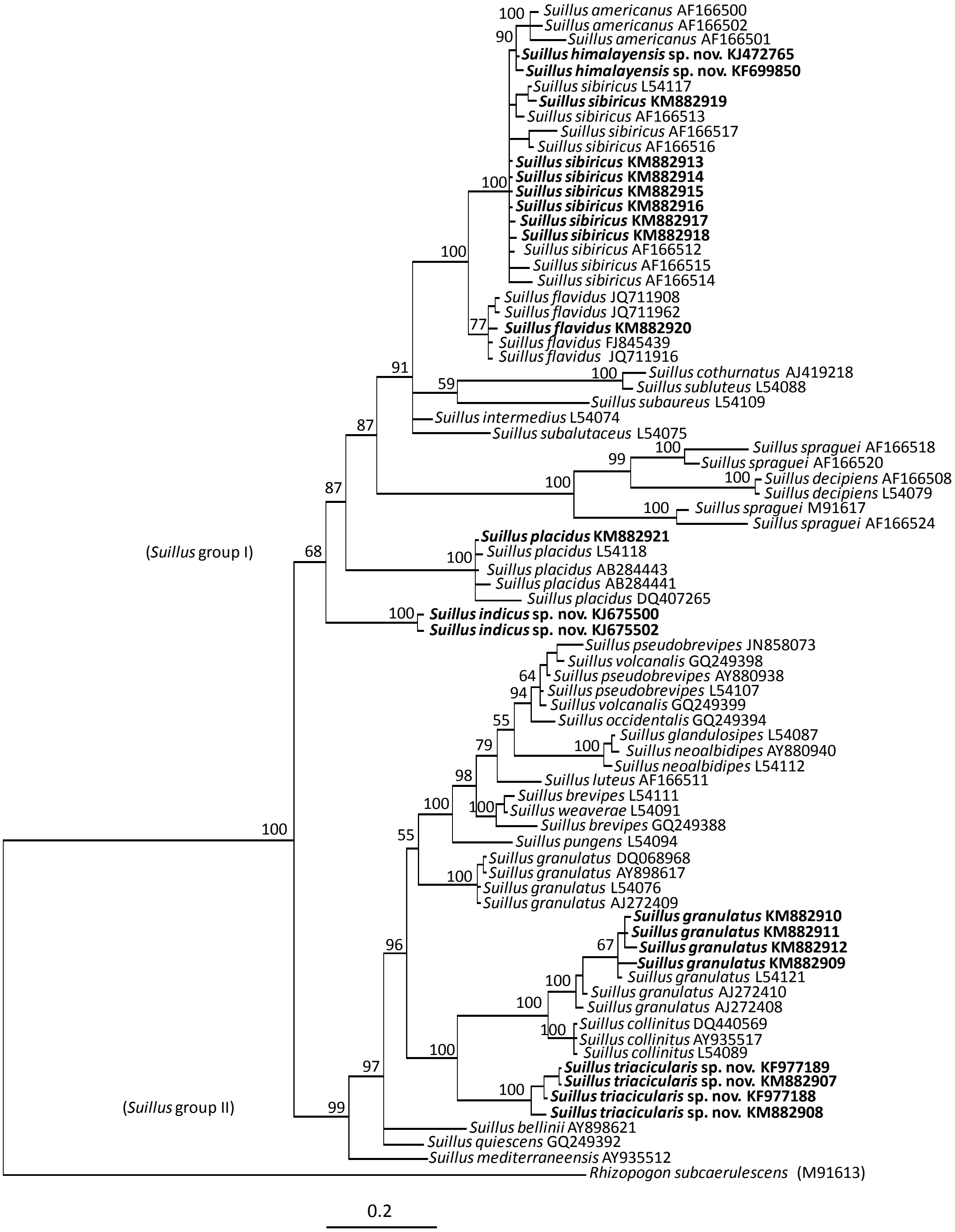
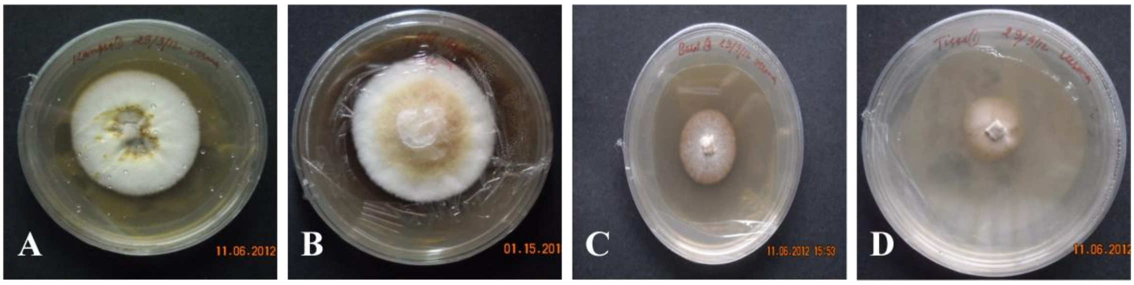


Fig. 4.17 Phylogenetic tree inferred from the bayesian analysis of the ITS region of *Suillus* species with *Rhizopogon subcaerulescens* as an out-group taxon. Bayesian posterior probability percentages (above 50%) are shown at the nodes of the tree. The scale represents the substitution rate. Bold indicates sequences derived in the present study.

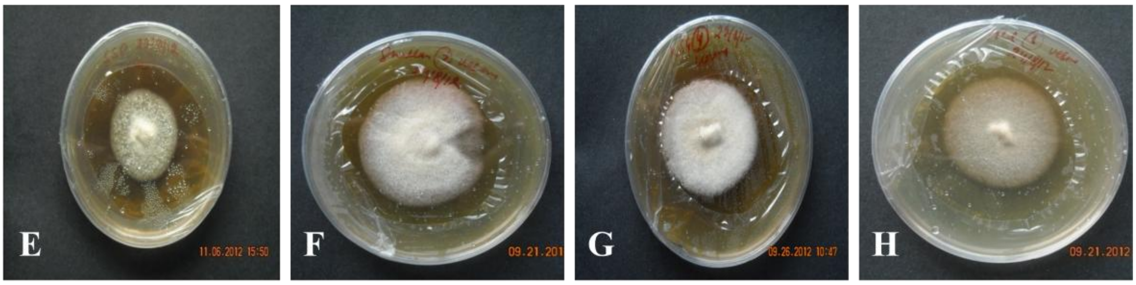
On the contrary, the ITS sequences of *S. granulatus* derived from Europe (L54076, AY898617, DQ068968, AJ272409, AJ272408, & AJ272410) and Asia (L54121, KM882909, KM882910, KM882911 & KM882912) are polyphyletic and sub-divided into two different clades (Fig. 4.17). Similarly, the ITS sequences of *Suillus spraguei* derived from American (M91617 and AF166524) and Chinese collections (AF166518 and AF166520) are also paraphyletic and sub-divided into two different subclades. Chinese *S. spraguei* isolates were found to be sister to American *S. decipiens* isolates (L54079 and AF166508) rather than American *S. spraguei* isolates.

4.2 Physiological characterization and *in vitro* evaluation of *Suillus* isolates

The *Suillus* cultures (Table 4.2 and Fig. 4.18) isolated from the sporocarps collected from different conifer forests of northwestern Himalayas were brought to the laboratory and maintained over 2% malt extract media by regular sub-culturing. The *Suillus* isolates were identified by molecular examination as described in section 4.1.5. Some physiological variables of different *Suillus* isolates obtained in the present study were evaluated in order to select suitable isolates for mass inoculum production. All of the isolates were examined for their radial growth, biomass yield, extracellular enzyme activities, and *in vitro* mycorrhizal abilities with *P. wallichiana* seedlings. Inter-specific as well as intra-specific variations were found in axenic fungal growth, extracellular enzyme activities and mycorrhizal abilities of different *Suillus* isolates.



Suillus triacicularis **SNW01** ***Suillus indica*** **SNW02** ***Suillus himalayensis*** **SNW03** ***Suillus granulatus*** **SNW04**



Suillus sibiricus **SNW05** ***Suillus sibiricus*** **SNW06** ***Suillus sibiricus*** **SNW07** ***Suillus sibiricus*** **SNW08**

Fig. 4.18 *Suillus* isolates (SNW01–SNW08) obtained from the sporocarps collected from different conifer forests of northwestern Himalayas

4.2.1 Radial growth and biomass yield

Radial growth of *Suillus* isolates examined in the present study ranged from 2.2 ± 0.2 cm in *S. granulatus* SNW04 to 5.0 ± 0.4 cm in *S. indicus* SNW02 (Fig. 4.19). 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. 4.20). *Suillus 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. 4.19 & Fig. 4.20). 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. *Suillus sibiricus* isolate SNW05 exhibited significantly lower radial growth and dry weight compared to other *S. sibiricus* isolates, whereas *S. sibiricus* isolate SNW06

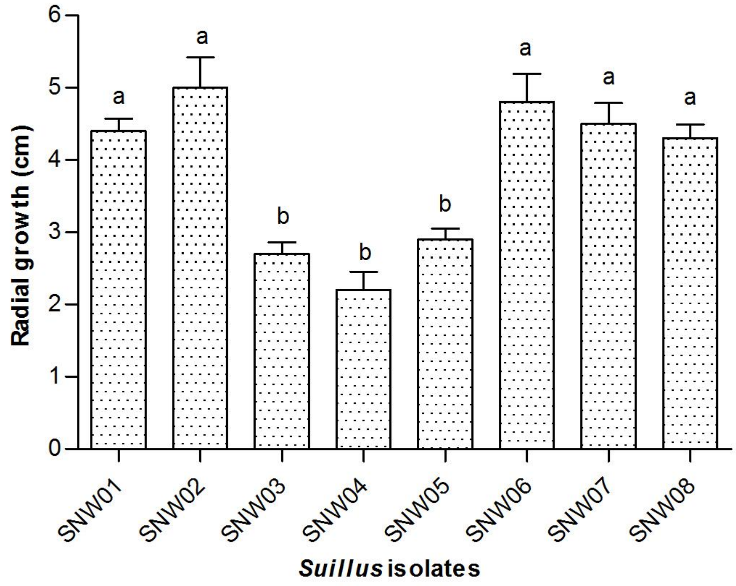


Fig. 4.19 Radial growth (cm) of *Suillus* isolates (SNW01–SNW08) on 2% malt extract agar medium as inferred from means of colony diameter. Different letters represent significant differences among the isolates according to the Tukey's test at P<0.05

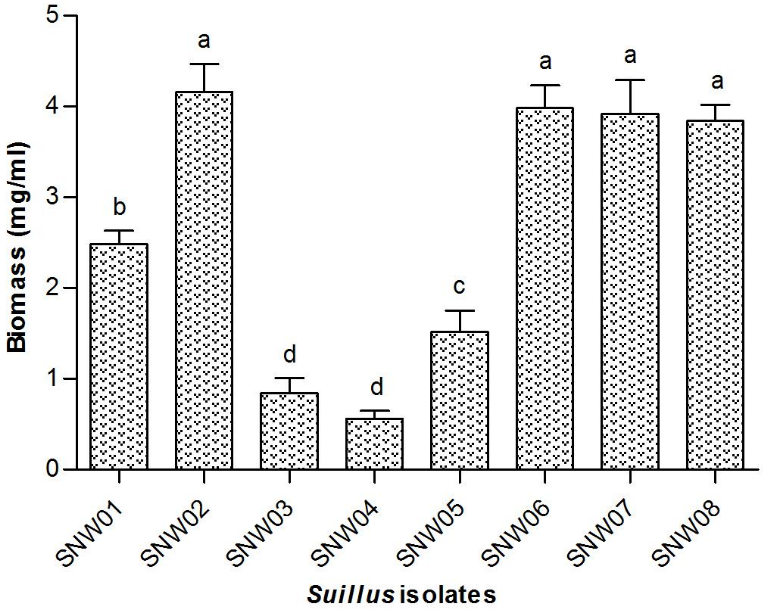


Fig. 4.20 Biomass yield (mg/ml) of *Suillus* isolates (SNW01–SNW08) in 2% malt extract broth medium as inferred from means of mycelium dry weight. Different letters represent significant differences among the isolates according to the Tukey's test at P<0.05

showed the highest growth. 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).

4.2.2 Extracellular Enzyme activities

Extracellular enzyme activities related to phosphorus and nitrogen uptake by ECM fungi, namely acid phosphatase, phytase, protease and chitinase were determined for all the eight *Suillus* isolates (Table 4.6). Intra-specific and inter-specific variations were also observed in the enzyme activities of different *Suillus* isolates of the present study.

Table 4.6 Extracellular enzyme activities of different *Suillus* isolates primarily involved in phosphorus and nitrogen uptake

***Suillus* isolate**	**Acid phosphatase activity (µM pNPg^{-1}h^{-1})**	**Phytase activity (µM Pig^{-1}h^{-1})**	**Protease activity (PUEs)**	**Chitinase activity (mM g^{-1}min^{-1})**
SNW01	48±11d	393±38c	0.88±0.11cd	2.14±0.25d
SNW02	89±05ab	359±51c	1.42±0.12ab	3.42±0.38c
SNW03	37±07d	810±59a	0.93±0.17cd	4.61±0.14b
SNW04	74±10bc	932±47a	1.24±0.09bc	6.51±0.37a
SNW05	49±06d	537±33b	0.76±0.16d	4.17±0.43bc
SNW06	107±12a	285±62c	1.55±0.10ab	1.54±0.23d
SNW07	82±09b	273±37c	1.74±0.19a	1.38±0.17d
SNW08	53±04cd	296±43c	1.18±0.14bc	1.92±0.27d

Values (Mean±SD) sharing similar letter within a single column are not significantly different at $P<0.05$.

4.2.2.1 Acid phosphatase (ACPase) activity

Inter-specific variation in acid phosphatase (ACPase) activities among different *Suillus* isolates was observed. ACPase activity of *Suillus* isolates ranged from 37 ± 7 to 107 ± 12 µM pNPg^{-1}h^{-1} (Fig. 4.21, Table 4.6), which was significantly higher in *S. sibiricus* SNW06 (107 ± 12 µM pNPg^{-1}h^{-1}). *Suillus himalayensis* SNW03, *S. triacicularis* SNW01 and *S. sibiricus* SNW05 exhibited lower ACPase activities, which was lowest in *S. himalayensis* SNW03 (37 ± 7 µM pNPg^{-1}h^{-1}). Similarly, intra-specific variation was found among different strains of *S. sibiricus*, which was observed to be 49 ± 6 µM pNPg^{-1}h^{-1} in *S. sibiricus* SNW05, 107 ± 12 µM pNPg^{-1}h^{-1} in *S. sibiricus* SNW06, 82 ± 9 µM pNPg^{-1}h^{-1} in *S. sibiricus* SNW07 and 53 ± 4 µM pNPg^{-1}h^{-1} in *S. sibiricus* SNW08.

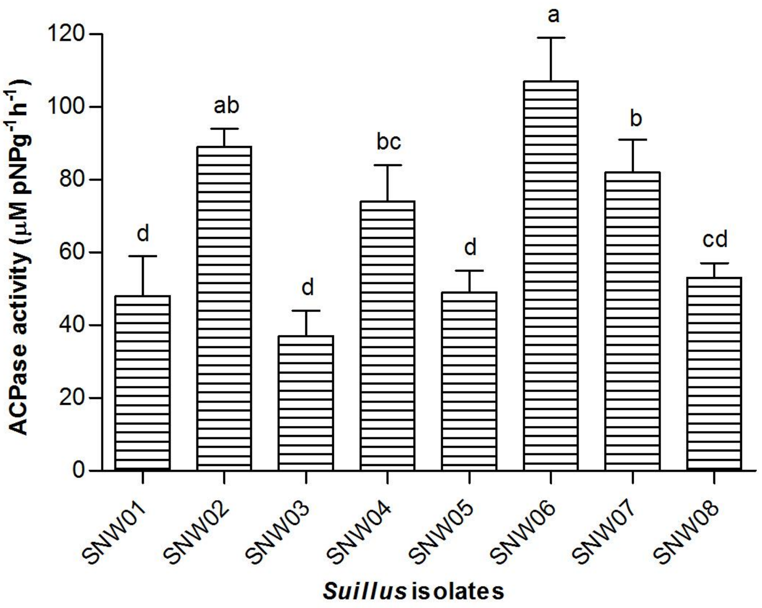


Fig. 4.21 Acid phosphatase activity (µM pNPg^{-1}h^{-1}) of different *Suillus* isolates (SNW01–SNW08). Different letters represent significant differences among the isolates according to the Tukey's test at $P<0.05$

4.2.2.2 Phytase activity

Phytase activity of *Suillus* isolates varied from 273 ± 37 to 932 ± 47 µM Pig^{-1}h^{-1} (Fig. 4.22, Table 4.6). *Suillus granulatus* SNW04 exhibited the maximum and *S. sibiricus* SNW07 exhibited the minimum phytase activity. *Suillus granulatus* SNW04 (932 ± 47 µM Pig^{-1}h^{-1}) and *S. himalayensis* SNW03 (810 ± 59 µM Pig^{-1}h^{-1}) showed significantly higher phytase activities, *S. sibiricus* SNW05 (537 ± 33 µM Pig^{-1}h^{-1}) showed intermediate value and rest of the *Suillus* isolates exhibited significantly lower phytase activities. Among the *S. sibiricus* strains, *S. sibiricus* SNW05 had significantly higher phytase activity as compared to other *S. sibiricus* strains.

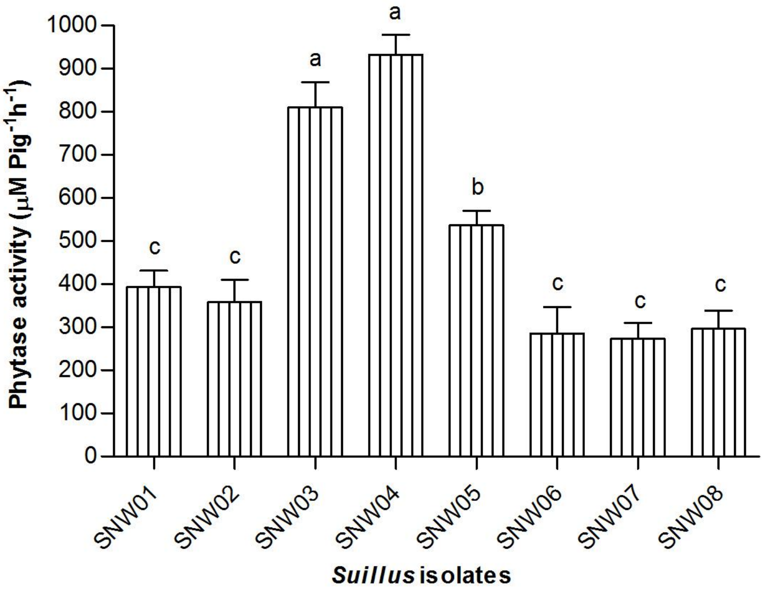


Fig. 4.22 Phytase activity (μM Pig^{-1}h^{-1}) of different *Suillus* isolates (SNW01–SNW08). Different letters represent significant differences among the isolates according to the Tukey's test at P<0.05

4.2.2.3 Protease activity

Protease activity ranged from 0.76 ± 0.16 to 1.74 ± 0.19 PUEs (Fig. 4.23, Table 4.6). *Suillus sibiricus* SNW07 showed significantly higher protease activity as compared to other *Suillus* isolates. The protease activity observed in *S. sibiricus* SNW07 was 1.74 ± 0.19 PUEs that was followed by *S. sibiricus* SNW06 (1.55 ± 0.1 PUEs) and *S. indicus* SNW02 (1.42 ± 0.12 PUEs), respectively. *Suillus sibiricus* SNW05 (0.76 ± 0.16 PUEs), *S. triacicularis* SNW01 (0.88 ± 0.11 PUEs) and *S. himalayensis* SNW03 (0.93 ± 0.17 PUEs) showed the significantly lower protease activities. Among the different *S. sibiricus* strains, *S. sibiricus* SNW07 exhibited significantly higher protease activities, *S. sibiricus* SNW06 and *S. sibiricus* SNW08 (1.18 ± 0.14 PUEs) exhibited intermediate values, whereas *S. sibiricus* SNW05 possessed significantly lower protease activities.

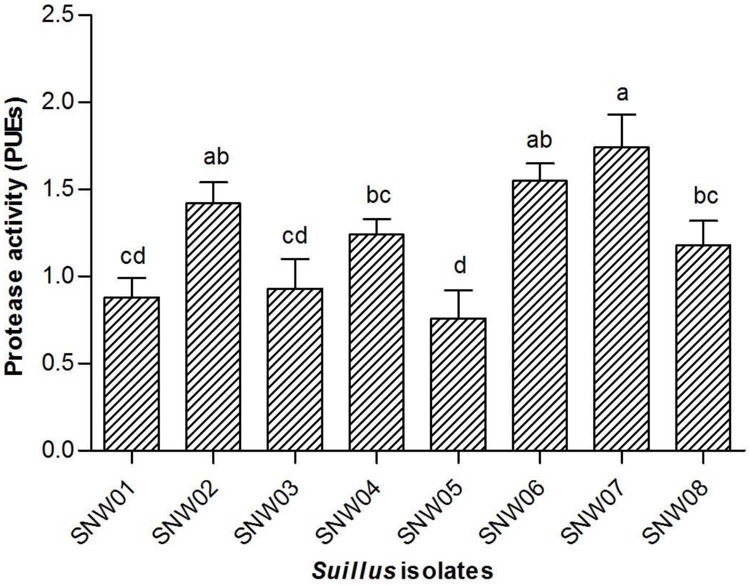


Fig. 4.23 Protease activity (PUEs) of different *Suillus* isolates (SNW01–SNW08). Different letters represent significant differences among the isolates according to the Tukey's test at P<0.05

4.2.2.4 Chitinase activity

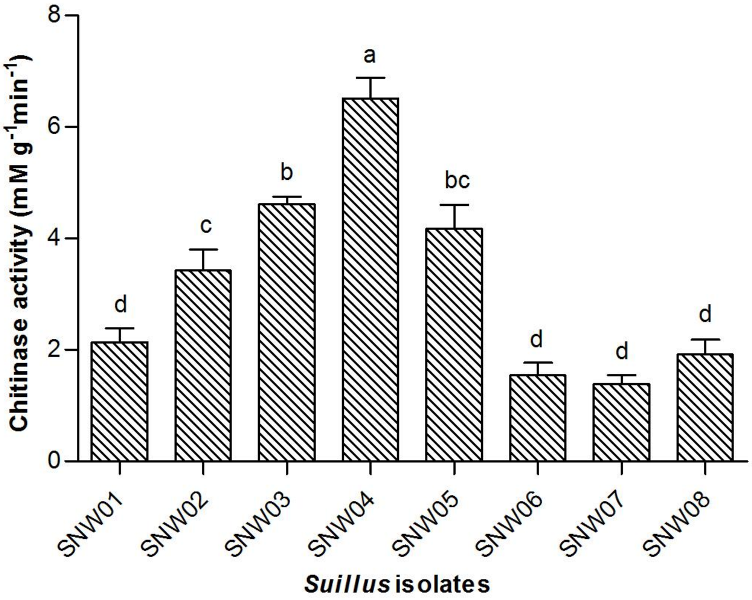


Fig. 4.24 Chitinase activity (mM g^{-1}min^{-1}) of different *Suillus* isolates (SNW01–SNW08). Different letters represent significant differences among the isolates according to the Tukey's test at P<0.05

Chitinase activity varied from 1.38 ± 0.17 mM $g^{-1}min^{-1}$ in *S. sibiricus* SNW07 to 6.51 ± 0.37 mM $g^{-1}min^{-1}$ in *S. granulatus* SNW04 isolate (Fig. 4.24, Table 4.6). Thus, the maximum chitinase activity was found in *S. granulatus* SNW04 that was followed by *S. himalayensis* SNW03 (4.61 ± 0.14 mM $g^{-1}min^{-1}$) and the minimum was observed in *S. sibiricus* SNW07. Like the phytase activity, *S. sibiricus* SNW05 (4.17 ± 0.43 mM $g^{-1}min^{-1}$) had significantly higher chitinase activity as compared to other three *S. sibiricus* strains.

4.2.3 Effects of *Suillus* isolates on the growth and nutrients content of *Pinus wallichiana* seedlings

4.2.3.1 *In vitro* mycorrhization

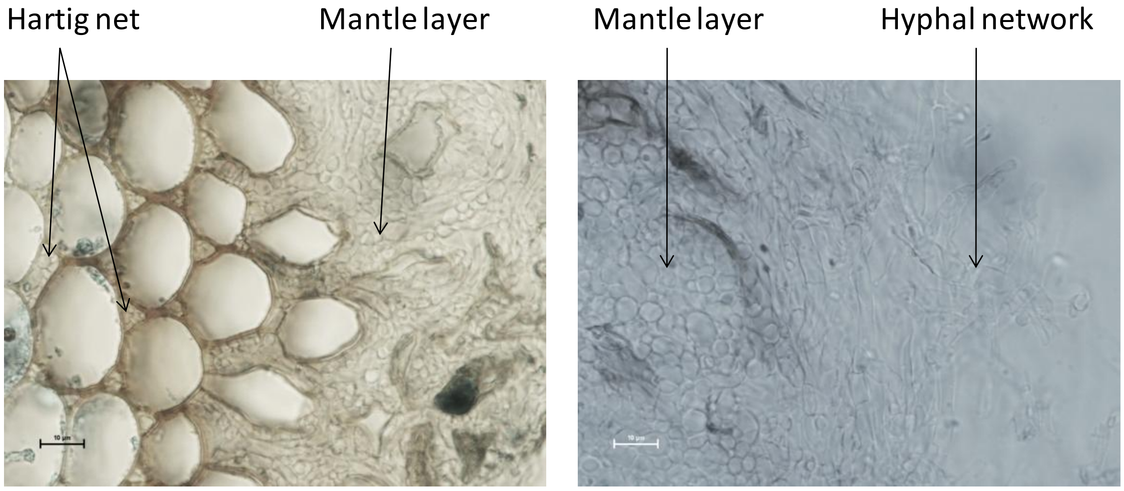


Fig. 4.25 Micrographs of pine root tip mycorrhizal with *Suillus* isolate after four months of the mycorrhization showing hartig net, mantle layer and emanating hyphal network

All the *Suillus* isolates tested colonized the roots of *P. wallichiana* seedlings irrespective of their natural host type. Micrographs of mycorrhizal root tips cross-section clearly showed the Hartig net, mantle layer and emanating hyphal network (Fig. 4.25). Mycorrhizal root colonization of *P. wallichiana* roots by different isolates of *Suillus* species varied considerably (Fig. 4.26) 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 *S. sibiricus* SNW05, *S. sibiricus* SNW06, *S. sibiricus* SNW07 and *S. sibiricus* SNW08, respectively. Among the different *Suillus* isolates, treatment with *S. sibiricus* SNW06 showed significantly higher mycorrhizal root colonization (71%) followed by *S. indicus* SNW02 (67%). On the contrary, *S. triacicularis* SNW01 showed significantly lower root colonization (23%). Treatments with other isolates showed intermediate colonization percentage values. No mycorrhizal colonization was observed on un-inoculated control seedlings.

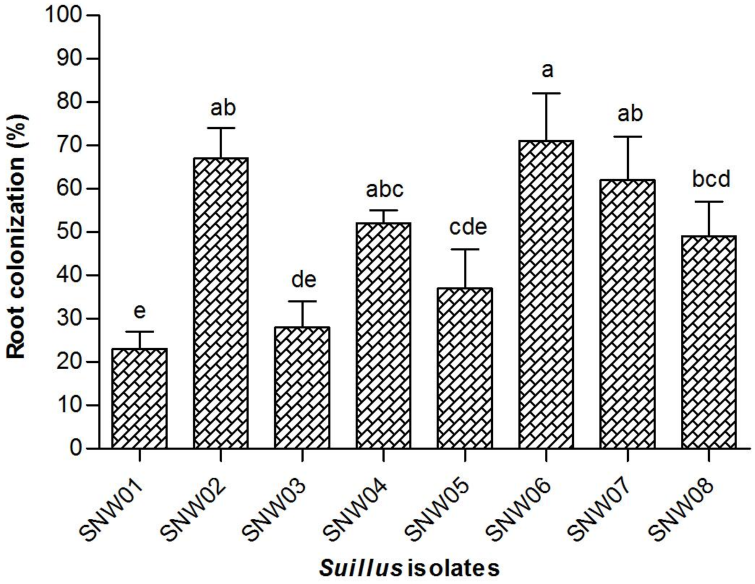


Fig. 4.26 Effect of inoculation with different *Suillus* isolates on ectomycorrhizal root colonization of blue pine (*Pinus wallichiana*) seedlings. Different letters represent significant differences among the isolates according to the Tukey's test at $P<0.05$

4.2.3.2 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 4.7. The results revealed that all the *Suillus* inoculants generally enhanced growth of *P. wallichiana* seedlings and improved the plant growth (seedling height and root length) and biomass (fresh and dry plant weight) compared to the uninoculated control treatment.

Table 4.7 Influence of inoculation with different *Suillus* isolates on growth and biomass of blue pine (*Pinus wallichiana*) seedlings after four months of the growth period

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

Values (Mean±SD) sharing similar letter within a single column are not significantly different at $P<0.05$.

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 51.4, 66.7, 43.6 and 52.3 % 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 its higher mycorrhizal colonization (71%) and acid phosphatase activity (107 μM pNPg^{-1}h^{-1}) in comparison to the other isolates. The minimum increase in the growth and biomass was observed in case of *S. himalayensis* SNW03, which comparatively exhibits poor mycorrhizal root colonization (28%) and lower acid phosphatase activity (37 μM pNPg^{-1}h^{-1}). Thus, the present study identifies *S. sibiricus* SNW06 and *S. indicus* SNW02 as efficient *Suillus* isolates for enhancement of growth and biomass of blue pine seedlings.

4.2.3.3 Influence on nutrients content of *P. wallichiana* seedlings

The content of nutrients in blue pine seedlings was modified by inoculation with *Suillus* inoculations and variable results were obtained depending on the fungus type and the nutrient considered (Table 4.8). The content of N was generally increased in all the *Suillus* treatments and this increase was highest with *S. sibiricus* SNW07 (16.1 ± 1.7 mg/g) followed by *S. sibiricus* SNW06 (15.6 ± 0.7 mg/g) and *S. indicus* SNW02 (15.3 ± 1.3 mg/g) treatments. *Suillus granulatus* SNW04 (12.8 ± 0.9 mg/g) and *S. sibiricus* SNW08 (12.0 ± 1.4 mg/g) exhibited the intermediate values, whereas *S. himalayensis* SNW03 (10.9 ± 0.6 mg/g), *S. triacicularis* SNW01 (10.5 ± 0.7 mg/g) and *S. sibiricus* SNW05 (10.3 ± 1.1 mg/g) showed significantly low increase in nitrogen content of the blue pine seedlings. The content of P was also increased by inoculation with all the *Suillus* isolates. The highest uptake of P was recorded in blue pine seedlings inoculated with *S. sibiricus* SNW06 (1.41 ± 0.37 mg/g) that was followed by *S. indicus* SNW02 (1.34 ± 0.28 mg/g). Treatments with *S. sibiricus* SNW07 (1.14 ± 0.2 mg/g), *S. granulatus* SNW04 (1.05 ± 0.3 mg/g), *S. sibiricus* SNW08 (0.99 ± 0.1 mg/g), *S. sibiricus* SNW05 (0.94 ± 0.18 mg/g) and *S. triacicularis* SNW01 (0.92 ± 0.23 mg/g) showed intermediate values with no significant variations. Significantly lower increase in P content of blue pine seedlings was observed in treatments with *S. himalayensis* SNW03 (0.81 ± 0.15 mg/g). Similarly, the content of K, Mg and Ca was generally increased in the seedlings treated with any of the *Suillus* isolates as compared to the control (un-inoculated seedlings). The increase was significantly higher when inoculated with *S. sibiricus* SNW06 and *S. indicus* SNW02 as compared to the all other treatments.

These results demonstrated that as compared with the control treatment, the content of nutrients in seedlings was generally increased by inoculation with all the isolates and this increase in nutrients content was variable for different isolates. The overall increase in nutrients content was significantly higher, when treated with *S. sibiricus* SNW06 followed by *S. indicus* SNW02 and *S. sibiricus* SNW07, respectively. On the contrary, significantly lower increase in total nutrients content was noticed in treatments with *S. sibiricus* SNW05, *S. triacicularis* SNW01 and *S. himalayensis* SNW03 isolates that was lowest in case of *S. himalayensis* SNW03.

Table 4.8 Influence of inoculation with different *Suillus* isolates on nutrients content of blue pine (*Pinus wallichiana*) seedlings after four months of the growth period

Treatments	**Nitrogen (mg/g seedling)**	**Phosphorus (m/g seedling)**	**Potassium (mg/g seedling)**	**Magnesium (mg/g seedling)**	**Calcium (mg/g seedling)**
Control	8.7±0.8d	0.71±0.17c	5.24±0.5b	0.89±0.29c	1.15±0.16b
SNW01	10.5±0.7cd	0.92±0.23abc	7.10±1.1ab	1.28±0.27abc	1.43±0.24ab
SNW02	15.3±1.3ab	1.34±0.28ab	8.25±0.9a	1.75±0.31ab	2.12±0.27a
SNW03	10.9±0.6cd	0.81±0.15bc	6.84±0.8ab	1.04±0.23bc	1.54±0.47ab
SNW04	12.8±0.9bc	1.05±0.30abc	7.82±1.2ab	1.62±0.15ab	1.93±0.31ab
SNW05	10.3±1.1cd	0.94±0.18abc	6.97±0.7ab	1.32±0.10abc	1.78±0.33ab
SNW06	15.6±0.7ab	1.41±0.37a	8.23±1.4a	1.87±0.17a	2.29±0.19a
SNW07	16.1±1.7a	1.14±0.20abc	7.59±0.4ab	1.69±0.34ab	2.05±0.42a
SNW08	12.0±1.4c	0.99±0.10abc	7.27±0.6ab	1.40±0.29abc	1.91±0.26ab

Values (Mean±SD) sharing similar letter within a single column are not significantly different at $P<0.05$.

Our experiments showed that inoculations with *Suillus* isolates increased seedling growth and nutrients content in relation with the uninoculated control treatment. On the basis of enzyme activities, mycorrhizal colonization, and the effects on seedlings growth, biomass and nutrients content; *S. sibiricus* SNW06 and *S. indicus* SNW02 were found to be the most effective and suitable *Suillus* isolates for the growth of *P. wallichiana* seedlings and therefore selected for mass inoculum production and field studies.

4.3 Optimization of culture conditions for mass inoculum production of selected *Suillus* isolates

4.3.1 Medium

Among the three different media [Modified Melin-Norkrans (MMN) agar media, Malt Extract (ME) agar (2% w/v) media and Potato dextrose agar (PDA) media] tested, the maximum radial growth for both the *Suillus* isolates (*Suillus indicus* SNW02 and *Suillus sibiricus* SNW06) was recorded when grown in malt extract medium (Table 4.9). *Suillus indicus* SNW02 (5.0 ± 0.4 cm) exhibited slightly higher growth as compared to *S. sibiricus* SNW06 (4.8 ± 0.3 cm). Both the *Suillus* isolates showed significantly lesser growth in PDA medium. Based upon these findings ME medium was selected for further experiments.

Table 4.9 Radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06 grown in different media

Medium (pH 5.5)	***S. indicus* SNW02 Colony diameter (cm)**	***S. sibiricus* SNW06 Colony diameter (cm)**
Modified Melin-Norkan's (MMN)	4.5±0.3ab	3.2±0.2b
2% Malt extract (ME)	**5.0±0.4a**	**4.8±0.3a**
Potato dextrose agar (PDA)	3.9±0.3b	2.7±0.5b

Values (Mean±SD) sharing similar letter within a single column are not significantly different at $P<0.05$.

4.3.2 Effects of varying concentrations of malt extract on the radial growth

Malt extract (ME) concentration was varied from 1–20 g/l and the radial growth of both the isolates was investigated (Fig. 4.27). The maximum growth of 5.9 ± 0.2 cm was observed at 5 g/l ME concentration for *S. indicus* SNW02 and 5.6 ± 0.2 cm at 2.5 g/l

ME concentration for *S. sibiricus* SNW06, which were then used for further experiments.

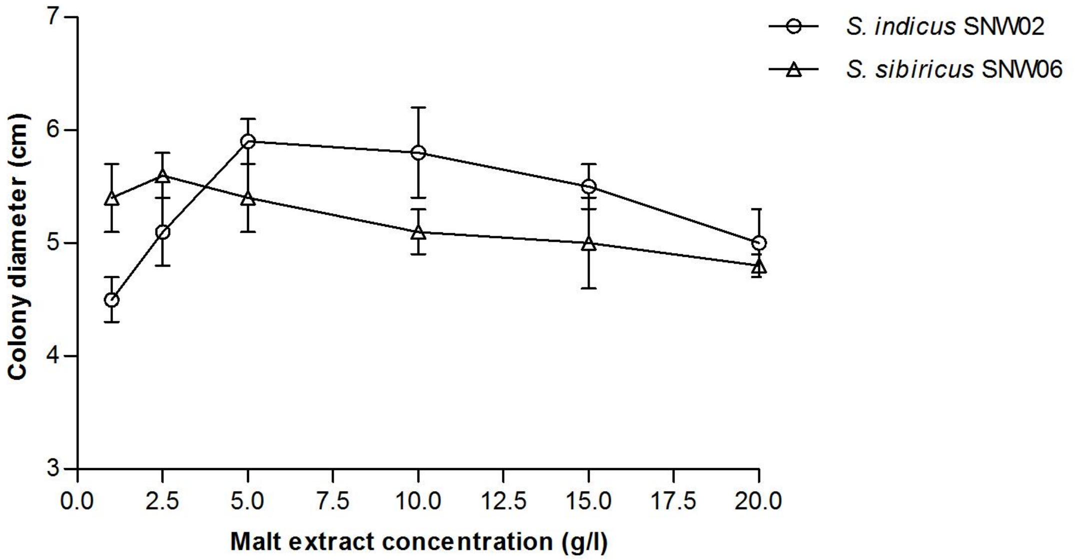


Fig. 4.27 Effects of varying concentrations of malt extract (g/l) on the radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06

4.3.3 Effects of various carbon sources

In the present study, effect of a number of carbon sources on growth of both the *Suillus* isolates was investigated and the results obtained are summarized in Fig. 4.28. Both the *Suillus* isolates showed a variable response to the carbon sources tested and preferred different carbon sources. For example, *S. indicus* SNW02 showed highest and almost similar radial growth whether the ME medium was supplemented with glucose (6.3 ± 0.3 cm) or sucrose (6.4 ± 0.2 cm), suggesting that for this ECM fungus either of these carbon sources can be used for mass cultivation. *Suillus indicus* SNW02 showed poor growth in the ME media containing sorbitol (0.7 ± 0.0 cm) and galactose (0.9 ± 0.0 cm) as carbon sources, suggesting that these carbon sources are the least preferable carbon sources for this ECM fungus as compared to the other carbon sources in the culture media. On the other hand, *S. sibiricus* SNW06 grew equally well in media containing galactose (5.7 ± 0.4 cm), glucose (5.8 ± 0.3 cm) or sucrose (5.7 ± 0.4 cm) as carbon sources and radial growth on these three carbon sources was higher than all other carbon sources tested. Intermediate growth values were obtained with fructose (5.3 ± 0.2 cm), lactose (4.8 ± 0.5 cm), maltose (4.5 ± 0.2 cm), mannitol (4.6 ± 0.3 cm) and sorbitol (4.8 ± 0.3 cm) as carbon source and minimum was observed with that of inositol (3.9 ± 0.5 cm). *Suillus sibiricus* SNW06 showed best radial growth (5.8 ± 0.3 cm) in the media containing glucose than all other carbon sources tested. These findings suggested that glucose can be used as a carbon source for both the *Suillus* isolates (*S. indicus* SNW02 *S. sibiricus* SNW06) for mass inoculum production, pine inoculation or other purposes.

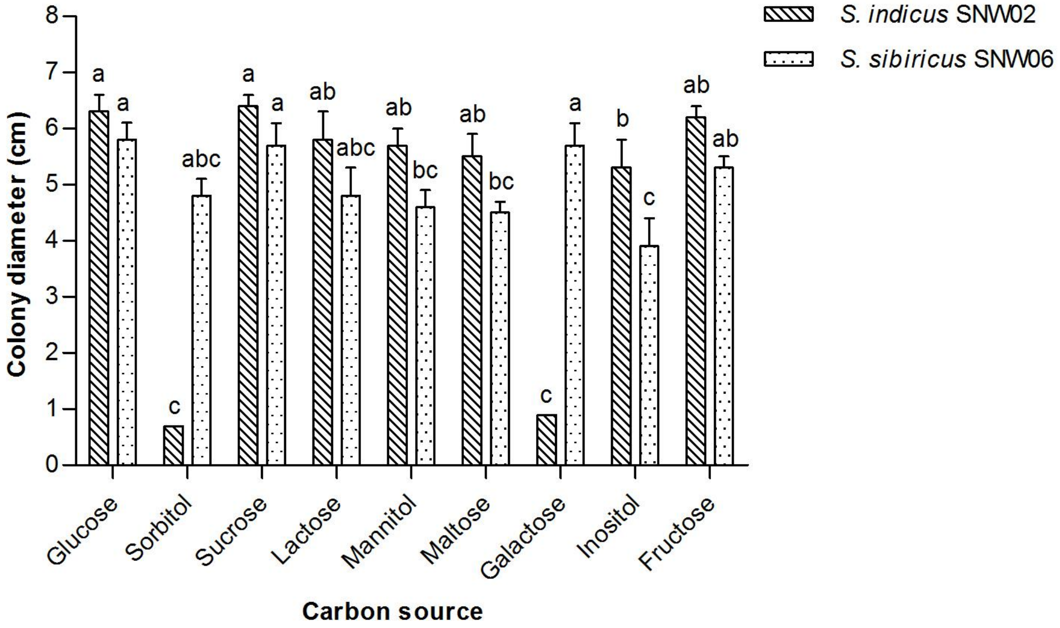


Fig. 4.28 Effects of various carbon sources on the radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06. Different letters above similar kind of bars represent significant differences among the carbon sources according to Tukey's test at $P<0.05$

4.3.4 Effects of varying concentrations of glucose

Both the *Suillus* isolates (*S. indicus* SNW02 and *S. sibiricus* SNW06) were capable of growing in the media containing very high glucose concentrations even up to 30 g/l, although the radial growth was minimal at this concentration (Fig. 4.29). In general, a large difference in radial growth in media with varying concentrations of glucose was observed (Fig. 4.29). In *S. indicus* SNW02, the radial growth first increased with an increase in glucose concentration and showed maximal radial growth at 20 g/l of glucose concentration, which was twice the glucose concentration of the control medium (10 g/l). The radial growth of *S. indicus* SNW02 in medium with 20 g/l glucose was 6.8 ± 0.3 cm, which was higher than in medium with 10 g/l glucose (6.3 ± 0.3 cm). The radial growth of *S. indicus* SNW02 decreased with further increase in the glucose concentration. Radial growth curve of *S. sibiricus* SNW06 was almost similar to that of *S. indicus* SNW02 and radial growth increased with increasing glucose concentrations, but the highest growth value (6.5 ± 0.2 cm) was observed at 15 g/l. With further increase in glucose concentration, radial growth decreased as in the case of *S. indicus* SNW02 when grown above 20 g/l glucose concentration.

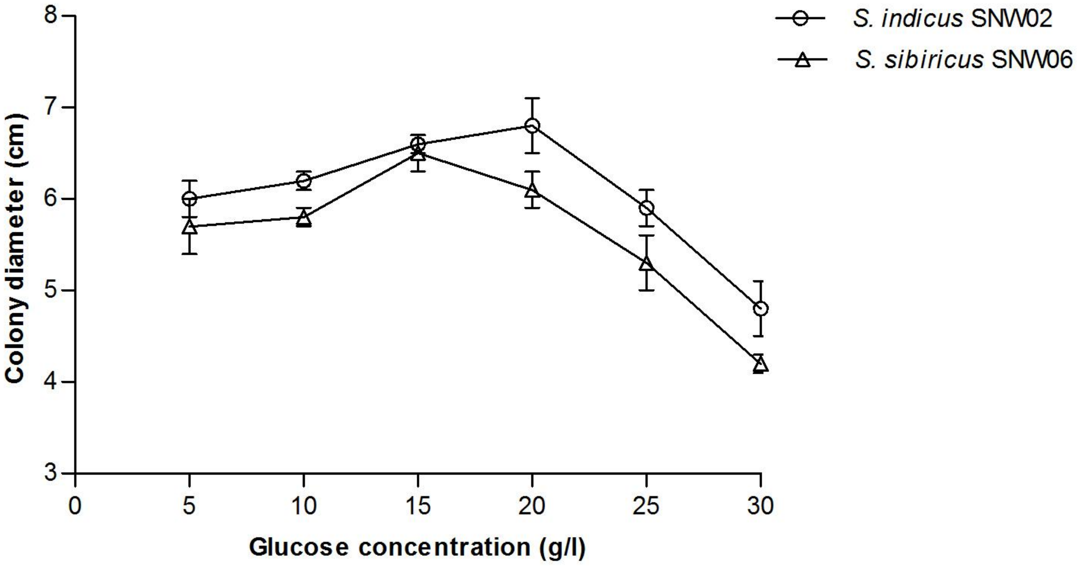


Fig. 4.29 Effects of varying concentrations of glucose (g/l) on the radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06

4.3.5 Effects of various nitrogen sources

The utilization of various sources of nitrogen such as, alanine, arginine, di-ammonium hydrogen phosphate, ammonium tartrate and potassium nitrate was tested for both *Suillus* isolates (*S. indicus* SNW02 and *S. sibiricus* SNW06). *Suillus indicus* SNW02 and *S. sibiricus* SNW06 showed no significant difference in radial growth with the five different nitrogen sources tested (Fig. 4.30). For both *Suillus* isolates, the radial growth in the medium with di-ammonium hydrogen phosphate appeared to be best than that in media with other nitrogen sources, but the difference was not very large.

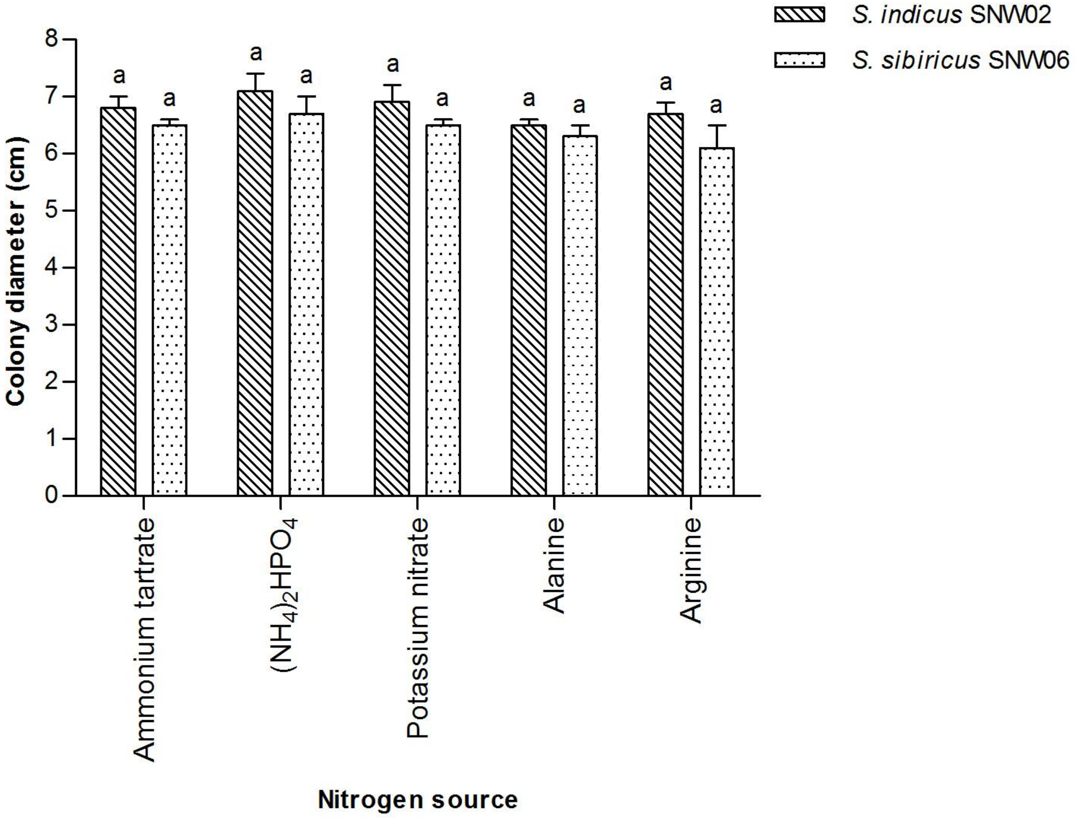


Fig. 4.30 Effects of various nitrogen sources on the radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06. Different letters above similar kind of bars represent significant differences among the nitrogen sources according to Tukey's test at $P<0.05$

Among all the five nitrogen sources tested, growth in the media with inorganic nitrogen (di-ammonium hydrogen phosphate and potassium nitrate) was higher than in media with organic nitrogen sources (alanine, arginine and ammonium tartrate). This suggested that these ECM fungi utilize inorganic sources of nitrogen better than organic combinations of nitrogen in the culture medium. Among the inorganic nitrogen sources (di-ammonium hydrogen phosphate and potassium nitrate), the radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06 was significantly better in the medium containing nitrogen in ammonical form than that in medium with nitrogen in nitrate form. These results revealed that ammonium is the preferable form of nitrogen for these ECM fungi.

4.3.6 Effects of varying concentrations of di-ammonium hydrogen phosphate

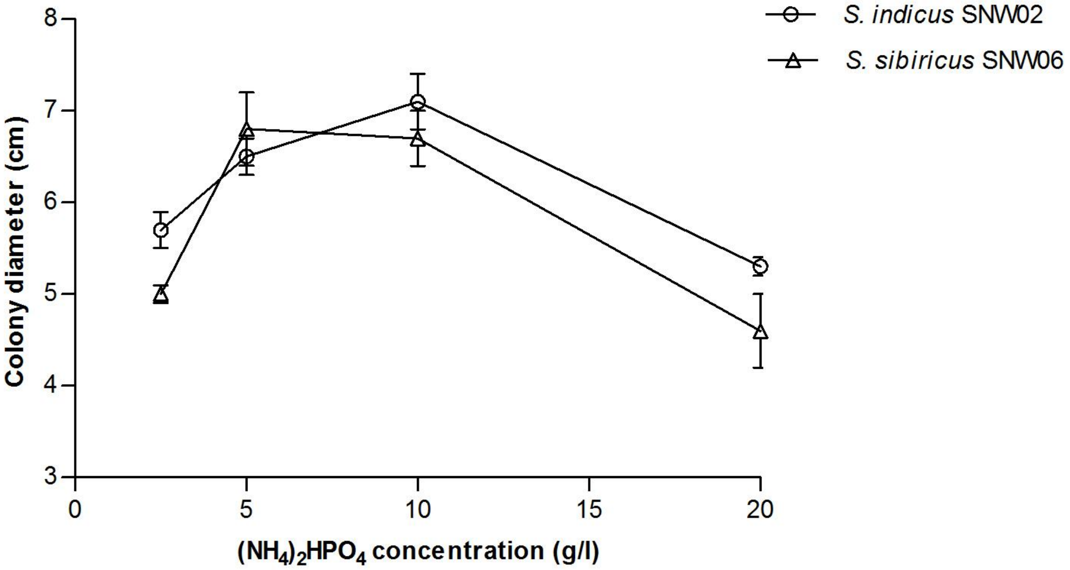


Fig. 4.31 Effects of varying concentrations of di-ammonium hydrogen phosphate (g/l) on the radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06

The concentration of di-Ammonium hydrogen phosphate [$(NH_4)_2HPO_4$] was varied from 2.5 g/l to 20.0 g/l in the media optimized so far for both the *Suillus* isolates and the radial growth was investigated after four weeks of incubation at 25ºC. The concentration of $(NH_4)_2HPO_4$ significantly affected the radial growth of both the isolates (Fig. 4.31). Generally, a large difference in radial growth in media with varying concentrations of $(NH_4)_2HPO_4$ was observed with glucose concentrations of 20 g/l and 15 g/l for *S. indicus* SNW02 and *S. sibiricus* SNW06, respectively. The optimum radial growth of 7.1 ± 0.3 cm at 10 g/l $(NH_4)_2HPO_4$ concentration for *S. indicus* SNW02 and 6.8 ± 0.4 cm at 5 g/l $(NH_4)_2HPO_4$ concentration for *S. sibiricus* SNW06 were observed, which were then used for the further experiments.

4.3.7 Effects of different adenosine concentrations on radial growth

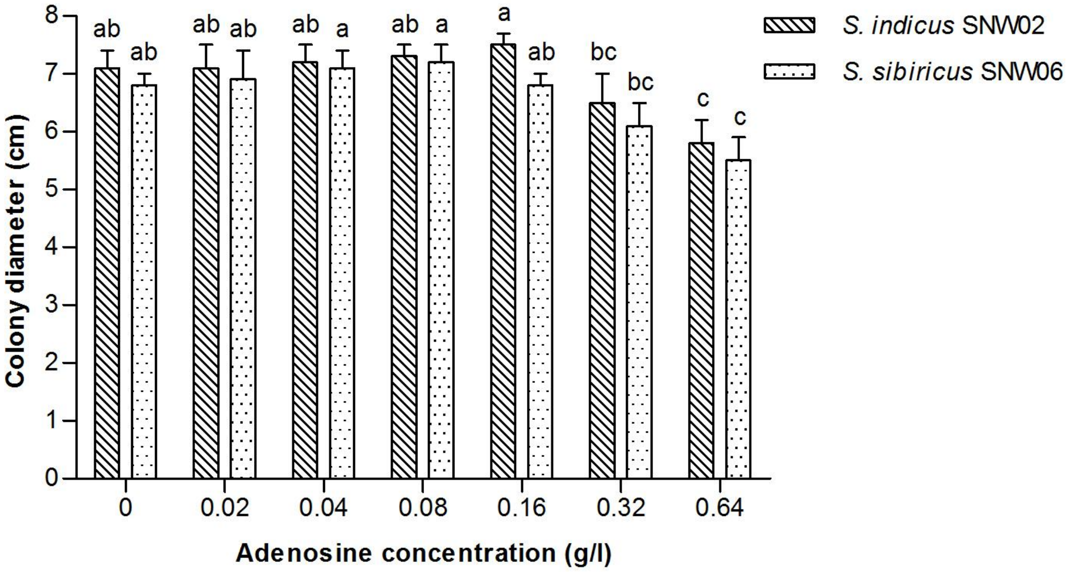


Fig. 4.32 Effects of different adenosine concentrations (g/l) on the radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06. Different letters above similar kind of bars represent significant differences among the adenosine concentrations according to the Tukey's test at $P<0.05$

Radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06 was measured on a range of concentrations of adenosine shown in Fig. 4.32. Addition of adenosine at low concentrations tends to increase the radial growth for both the *Suillus* isolates. For *S. indicus* SNW02, an increase in growth was observed when cultured in media containing 0.02 to 0.16 g/l adenosine. The highest growth (7.5 ± 0.20 cm) was found at 0.16 g/l adenosine. Also, the radial growth of *S. sibiricus* SNW06 increased in the presence of adenosine concentrations ranging from 0.02 to 0.08 g/l. The highest growth value (7.2 ±

0.3 cm) was obtained when the adenosine concentration in the medium was at 0.08 g/l. The results showed that no stimulatory effect on radial growth was found at adenosine concentration ≥0.32 g/l and the radial growth of both the isolates was retarded at these concentrations.

4.3.8 Temperature

The radial growth of both the selected *Suillus* isolates was measured at different temperatures (15°C–40°C, Fig. 4.33). The maximum radial growth (7.5 ± 0.2 cm) for *S. indicus* SNW02 was observed at 25°C and for *S. sibiricus* SNW06 (7.3 ± 0.3 cm) at 30°C. Both the isolates grew well between 15–35°C but *S. indicus* SNW02 was incapable of growing at 40°C, whereas *S. sibiricus* SNW06 showed much retarded growth at 40°C. Hence, the *Suillus* isolates tested are mesophilic in nature.

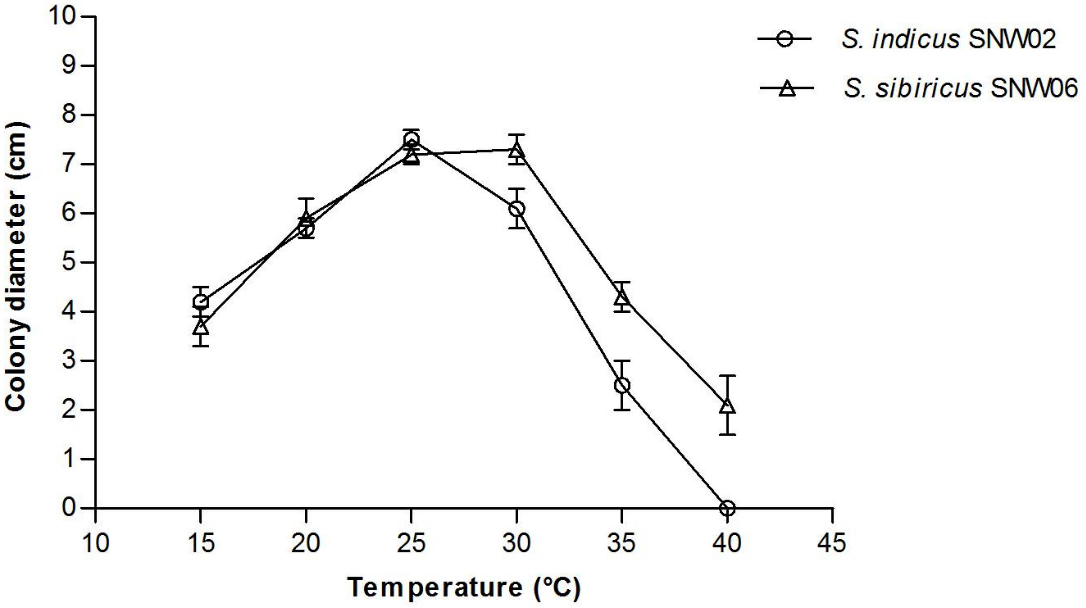


Fig. 4.33 Effect of incubation temperature on radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06

4.3.9 pH

The effect of varying pH (4.5–7.5) on the growth of both the *Suillus* isolates was investigated (Fig. 4.34). These ECM fungi can grow in a wide range of pH (4.5–7.5), but the maximum growth was observed in acidic pH for both the isolates. *Suillus indicus* SNW02 exhibited maximum radial growth (7.9 ± 0.2 cm) at pH 5.0, although it exhibited good growth between pH 5.0–5.5. *Suillus sibiricus* SNW06 grew well in pH range 5.5–6.5 with maximum (7.4 ± 0.5 cm) growth being at pH 6.0. Towards more acidic and more alkaline conditions, both the *Suillus* isolates showed inhibition in the radial growth.

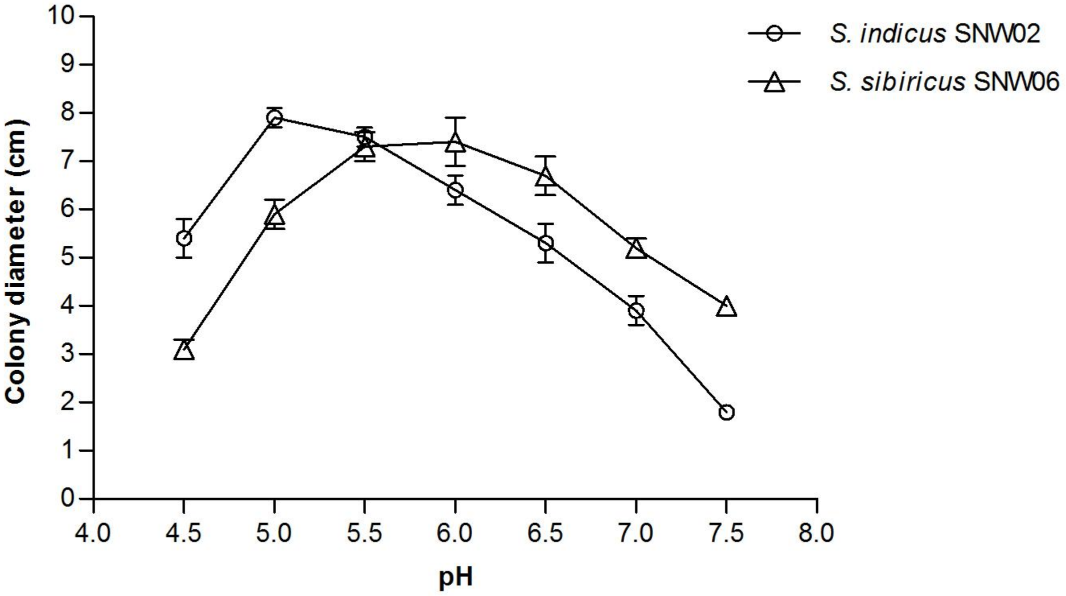


Fig. 4.34 Effect of pH of medium on radial growth of *S. indicus* SNW02 and *S. sibiricus* SNW06

Finally, the optimization of various parameters for the growth of both the *Suillus* isolates resulted in a significant increase in the radial growth (Table 4.10). For *S. indicus* SNW02, 1.58 fold increase was obtained in radial growth than the initial growth in 2% ME medium. In case of *S. sibiricus* SNW06, 1.54 fold increase was achieved in radial growth as compared to the initial growth in 2% ME medium.

4.3.10 Mycelial growth of selected *Suillus* species in optimized broth media

Table 4.10 Radial growth and biomass yield of *S. indicus* SNW02 and *S. sibiricus* SNW06 in basal 2% malt extract (ME) medium and respective optimized media

Medium	***S. indicus* SNW02 Colony diameter (cm)**	***S. sibiricus* SNW06 Colony diameter (cm)**	***S. indicus* SNW02 Biomass yield (mg/ml)**	***S. sibiricus* SNW06 Biomass yield (mg/ml)**
2% ME medium	5.0±0.4b	4.8±0.3b	4.16±0.31b	3.98±0.25b
Optimized medium	**7.9±0.2a**	**7.4±0.5a**	**6.07±0.38a**	**5.53±0.42a**

Values (Mean±SD) sharing similar letter within a single column are not significantly different at $P<0.05$.

Further down the study, mycelial growth of both the *Suillus* isolates in the respective optimized broth media (OM1 and OM2) was investigated (Table 4.10) and compared to the initial biomass yield. It was found that biomass yield increased from 4.16 ± 0.31 mg/ml to 6.07 ± 0.38 mg/ml in *S. indicus* SNW02, thus showing 1.46 fold increase in biomass yield. Similarly, biomass yield of *S. sibiricus* SNW06 increased 1.39 fold from 3.98 ± 0.25 mg/ml initial yield to 5.53 ± 0.42 mg/ml final biomass yield. Thus, the optimization of culture conditions have remarkably increased the radial growth as well as the biomass yield of both the selected *Suillus* isolates, which is crucial for mass inoculum production in forestry practices.

4.4 Influence of selected *Suillus* species and biochar amendment on plant growth and rhizospheric properties

4.4.1 Characteristics of Pine needle Biochar

The biochar (BC) used in the present study was manufactured from blue pine (*P. wallichiana*) needles collected from a nearby forest site (Fig. 4.35A). Biochar was produced by pyrolysis at 650°C. The BC obtained (Fig. 4.35B) was ground to fine particles before applying it to the soil. The powdered biochar was then applied to the soil at 2% ratio (w/w). The analytical characteristics of the BC were determined in this study. The BC was slightly alkaline in nature with pH 7.8 and exhibited high cation exchange capacity (CEC=240 cmol/kg). Total N estimated in biochar was .33 %. Total organic carbon content of biochar was very high (90.8%) with C/N ratio of 275. Total P (310 mg/kg) and available P (5.84 mg/kg) content were comparatively low, which may be due to high pyrolysis temperature (650°C).

Fig. 4.35 A. A heap of *Pinus wallichiana* needles collected from the blue pine (*Pinus wallichiana*) forest **B.** Biochar obtained by pyrolysis of *Pinus wallichiana* needles

4.4.2 Ectomycorrhizae of *Suillus* species on *P. wallichiana* seedlings

A nursery trial of *Pinus wallichiana* seedlings was established at Deeb (1830 m elevation), Kumarsain, Shimla, Himachal Pradesh, India (Fig. 4.36). The experiment was conducted under open field conditions in randomized block designed manner. Seedlings were grown for nine months after inoculation and then harvested destructively.

Root systems of *P. wallichiana* seedlings colonized with *S. indicus* SNW02 and *S. sibiricus* SNW06 were washed properly with tap water to remove soil particles and are shown in Fig. 4.37. Root systems of both the *Suillus* species consisted of abundant and randomly distributed highly dichotomous mycorrhizal systems. These mycorrhizal systems differed remarkably in morphological and anatomical details, which are described ahead:

Fig. 4.36 A view of experimental site showing *Pinus wallichiana* seedlings growing in polypropylene bags

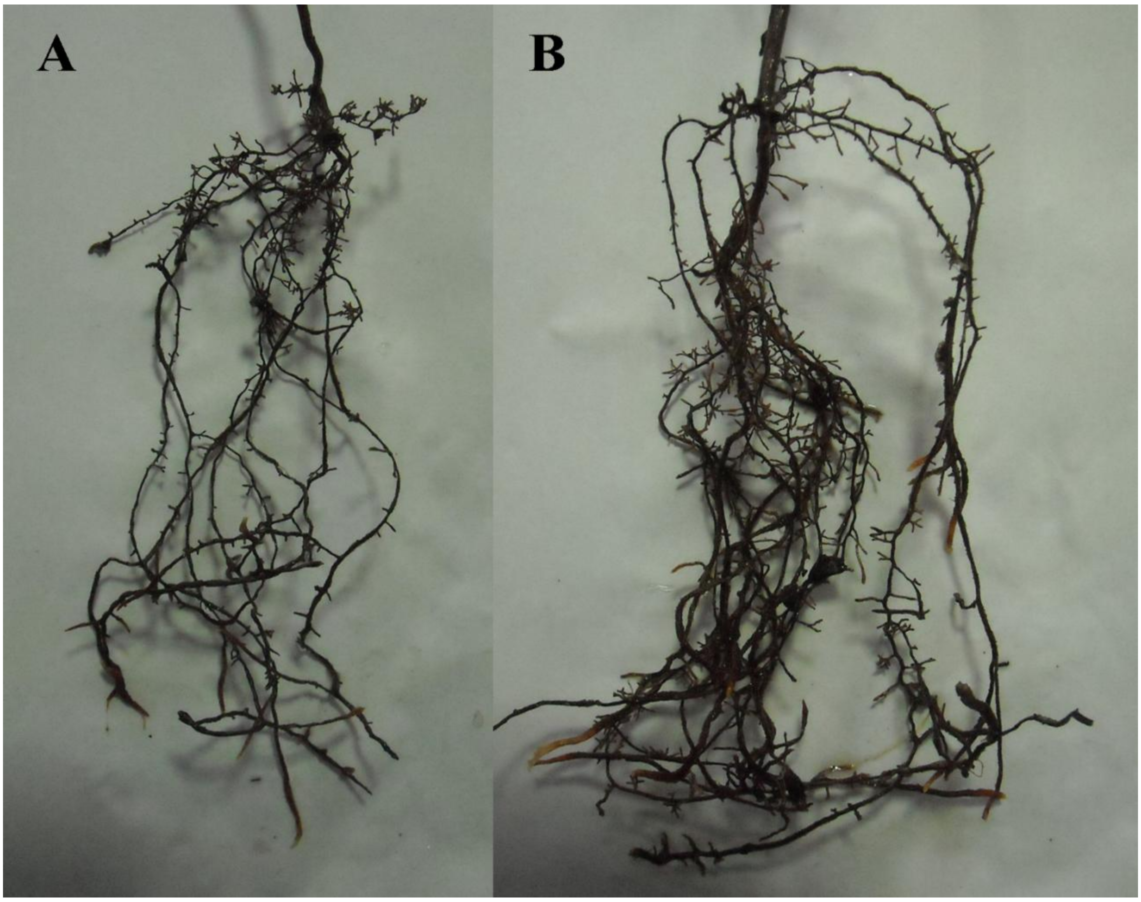


Fig. 4.37 Root systems of blue pine (*Pinus wallichiana*) seedlings at the end of the nursery stage: A. Roots colonized with *S. indicus* SNW02 B. Roots colonized with *S. sibiricus* SNW06

Ectomycorrhizae of *Suillus indicus* on *Pinus wallichiana*:

Morphology of mycorrhizal systems: Mycorrhizal systems highly dichotomous, abundant and dense (Figs. 4.38A–4.38B); systems ≤ 4.5 mm long, ≤ 3.0 mm broad, with ≤ 0.3 mm thick basal stalk; color varies considerably with the development, yellowish brown in the youngest to dark blackish brown in the oldest systems (Figs. 4.38A–4.38C). Unramified ends ≤ 1.6 mm long, ≤ 0.4 mm in diameter; young tips yellowish brown to brownish, straight, club shaped (Fig. 4.38B); mature tips dark blackish brown, cylindrical and constricted between older and younger parts (Fig. 4.38C). The growing tip apices of unramified ends round and pale yellow; mantle surface of unramified ends loosely woolly and the host tissue under the surface invisible.

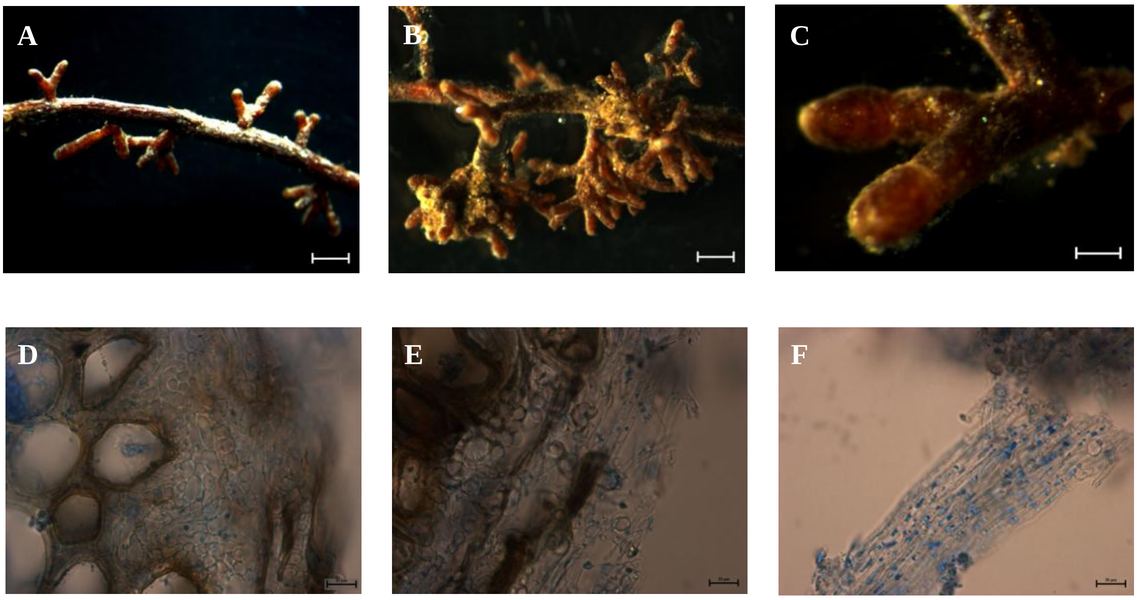


Fig. 4.38 *Suillus indicus* ectomycorrhizae on *Pinus wallichiana*: **A&B.** Simple to highly dichotomous mycorrhizal systems; **C.** Mature constricted unramified tips; **D&E.** Anatomy of mantle layers; **D.** Inner mantle layer- pseudoparenchymatous with roundish to angular cells **E.** Outer mantle layer- plectenchymatous with undifferentiated hyphae, type F, mounds of roundish cells and inflated cells present; **F.** Rhizomorph, type A, septa and clamps common. Scale bars: A–C=200 µm; D–F= 10 µm

Anatomy of mantle: The plectenchymatous outer mantle layer (Fig. 4.38E) is of the F type (Agerer, 1991); hyphae 3.0–7.0 µm in diameter, with inflated cells and some groups of round, swollen cells above the undifferentiated mantle; hyphal walls smooth, thin, as thick as hyphal septa, no matrix material and clamps observed, suilloid crystals present. Inner mantle layer (Fig. 4.38D) pseudoparenchymatic, 6–10 cell layers thick, no matrix material observed and numerous tannin cells present; cells mostly angular , ≤ 12 µm in size. Hartig net of common type extending up to the endodermis and small globular haustoria found are 10.0–18.0 µm in diameter.

Emanating elements: Uniform rhizomorphs (Fig. 4.38F) with loosely woven undifferentiated vegetative hyphae, observed under light microscope and are of type A (Agerer, 1991); hyphae 1.5 × 4.0 µm in diameter; numerous suilloid crystals present all over the hyphal surface; rhizomorphs ≤ 50 µm in diameter with few hyphae emanating from the surface; septa and clamps common; H-shaped anastomoses present.

Ectomycorrhizae of *Suillus sibiricus* on *Pinus wallichiana*:

Morphology of mycorrhizal systems: Mycorrhizal systems simple to highly dichotomous, abundant and dense (Figs. 4.39A–4.39B); systems ≤ 2.5 mm long, ≤ 1.2 mm broad, with ≤ 0.2 mm thick basal stalk; color varies considerably with the development, light brown to reddish brown to dark honey brown in the oldest systems. Unramified ends ≤ 1.5 mm long, ≤ 0.3 mm in diameter; tips light brown to reddish brown, straight, club shaped (Fig. 4.39C); mature tips dark honey brown. Mantle surface heavily cottony and the host tissue invisible under the sheath (Fig. 4.39C).

Anatomy of mantle: Outer mantle (Fig. 4.39D) is of type L (Agerer, 1991), pseudoparenchymatous, with angular to roundish cells, cells hyaline to pale yellowish, ≤ 9 µm in diameter. Inner mantle (Fig. 4.39D) also pseudoparenchymatous with epidermoid puzzle like structures, cells ≤ 7.0 µm in diameter, cell contents clear, no matrix material observed and numerous tannin cells present. Hartig net is of common type extending up to the endodermis and small globular haustoria were also observed (Fig. 4.39F).

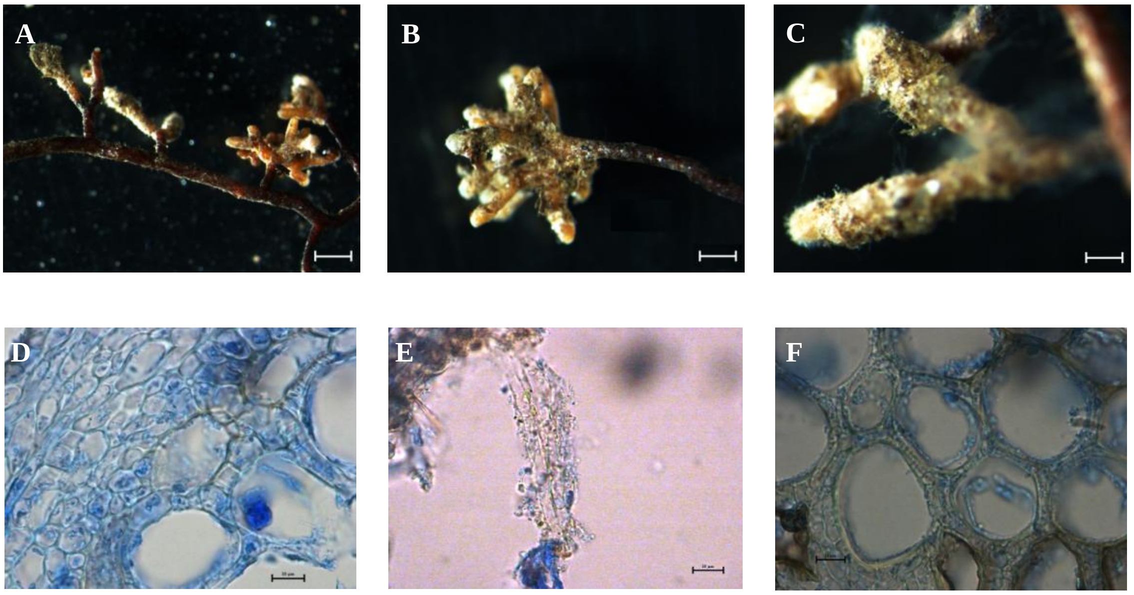


Fig. 4.39 *Suillus sibiricus* ectomycorrhizae on *Pinus wallichiana*: **A,B&C.** Simple to highly dichotomous mycorrhizal systems; **D.** Anatomy of mantle layers: Outer mantle-pseudoparenchymatous, type L, with angular to roundish cells, Inner mantle-pseudoparenchymatous with epidermoid puzzle like structures; **E.** Emanating hyphae; **F.** Common Hartig net with globular haustoria. Scale bars: A–C=200 µm; D–F= 10 µm

Emanating elements: Emanating hyphae (Fig. 4.39E), very common giving cottony appearance to the systems, straight, hyphae ≤ 4.5 µm in diameter, 55–70 µm in length, clamps present at septa.

4.4.3 Ectomycorrhizal colonization and plant growth of *P. wallichiana* seedlings

No colonization was observed in uninoculated treatments. Among the mycorrhizal treatments, biochar application showed slight increase in mycorrhizal root colonization percentage in comparison to the non-biochar treatments (Table 4.11). BC+ *S. sibiricus* SNW06 treatment showed the highest root colonization as compared to all other treatments. Also as found with *in vitro* studies, *S. indicus* SNW02 exhibits lower root colonization value than *S. sibiricus* SNW06. Although BC + *S. indicus* SNW02 and BC + *S. sibiricus* SNW06 tended to increase mycorrhizal root colonization as compared to single mycorrhizal treatments, but there were no significant differences in root colonization percentages.

Seedling heights, root length, shoot biomass and root biomass varied significantly across all the treatments. In single mycorrhizal treatments, inoculation with *S. sibiricus* SNW06 showed higher seedling height, root length, shoot biomass and root biomass than those inoculated with *S. indicus* SNW02 isolate. As compared to the control, single mycorrhizal treatments with *S. indicus* SNW02 and *S. sibiricus* SNW06 enhanced seedlings height by 40.2% and 46.7%, root length by 42.1% and 48.4%, shoot biomass by 41.0% and 49.8%, and root biomass by 43.2% and 50.6%, respectively.

Table 4.11 Effects of mycorrhizal inoculants and biochar amendment on mycorrhizal root colonization and plant growth of *Pinus wallichiana* seedlings

Treatments		**Mycorrhizal root colonization (%)**	**Seedling height (cm)**	**Root length (cm)**	**Shoot biomass (mg/plant)**	**Root biomass (mg/plant)**
No biochar	Control	-	09.2±0.7f	12.6±1.2e	239±12d	176±15b
	S. indicus SNW02	74±6.0a	12.9±0.5d	17.9±0.9c	337±37b	252±11a
	S. sibiricus SNW06	82±4.0a	13.5±1.2c	18.7±1.1bc	358±44b	265±18a
2% biochar	BC	-	10.8±1.1e	14.3±0.7d	288±25c	198±07b
	BC+ *S. indicus* SNW02	79±8.0a	15.3±0.8b	19.8±0.8ab	401±28a	269±22a
	BC+ *S. sibiricus* SNW06	85±3.0a	16.4±1.4a	20.3±1.5a	422±36a	277±20a

Values (Mean±SD) sharing similar letter within a single column are not significantly different at $P<0.05$.

Biochar treatment significantly increased the growth and biomass of blue pine seedlings, but the greater improvement was observed when treated both with biochar and the *Suillus* inoculants. In dual treatments, BC + *S. sibiricus* SNW06 treatment showed higher enhancement in plant height, root length, shoot biomass and root biomass of the seedlings followed by BC + *S. indicus* SNW02 treated seedlings. Thus, BC + *S. sibiricus* SNW06 treated seedlings showed higher increase in the growth and biomass of blue pine seedlings as compared to the all other treatments. The seedling height, root length, shoot biomass and root biomass were increased by 78.3%, 61.1%, 76.6% and 57.4% as compared to un-inoculated control and 51.9%, 42.0%, 46.5% and 39.9%, respectively as compared to biochar amended seedlings only.

4.4.4 Nutrients content of blue pine (*P. wallichiana*) seedlings

Nutrients (N, P, K) contents in blue pine seedlings were estimated to study the effects of biochar, selected *Suillus* isolates, and biochar plus *Suillus* isolates treatments on the nutrients uptake by plants. Nitrogen content of seedlings varied from 10.2 ± 1.1 to 17.3 ± 1.6 mg/g seedling, phosphorus content from 1.17 ± 0.23 to 2.26 ± 0.32 mg/g seedling and potassium content from 6.14 ± 0.8 to 9.36 ± 1.5 mg/g seedling in different treatments (Table 4.12). Plants inoculated with *S. sibiricus* SNW06 showed higher nutrient content than plants inoculated with *S. indicus* SNW02. Biochar treatment enhanced the N, P and K content of plants by 12.1%, 23.1% and 10.9%, respectively as compared to the un-inoculated control treatment. Among all the treatments, BC + *S. sibiricus* SNW06 treatment showed significantly higher nutrients content followed by BC + *S. indicus* SNW02 treatment. BC + *S. sibiricus* SNW06 showed significantly higher increase in nitrogen and phosphorus content of the seedlings as compared to BC + *S. indicus* SNW02 treatment, whereas increase in potassium content was not significantly different.

Table 4.12 Effects of mycorrhizal inoculants and biochar amendment on nutrients content of *Pinus wallichiana* seedlings

Treatments		Nitrogen (mg/g plant)	Phosphorus (mg/g plant)	Potassium (mg/g plant)
No biochar	Control	10.2±1.1e	1.17±0.23e	6.14±0.8e
	S. indicus SNW02	15.75±0.8c	1.59±0.11c	8.53±0.7c
	S. sibiricus SNW06	16.57±1.4b	1.78±0.19b	8.84±1.4b
2% biochar	BC	11.43±0.9d	1.44±0.21d	6.81±1.2d
	BC+ *S. indicus* SNW02	16.80±1.3b	1.85±0.13b	9.22±0.9a
	BC+ *S. sibiricus* SNW06	17.30±1.6a	2.26±0.32a	9.36±1.5a

Values (Mean±SD) sharing similar letter within a single column are not significantly different at P<0.05.

4.4.5 Physico-chemical properties of Soil

pH and cation exchange conductivity (CEC):

Only the BC amendment slightly increased the soil pH, whereas mycorrhizal inoculation with *S. indicus* SNW02 and *S. sibiricus* SNW06 decreased the soil pH (Table 4.13). This may be attributed to the formation of low molecular weight organic acids by mycorrhizal fungi. The decrease in the soil pH was significantly higher in single mycorrhizal treatments as compared to combined treatments with biochar. In dual treatments of biochar and mycorrhizal fungi the decrease in soil pH was comparatively low.

Both, mycorrhizal inoculation and biochar amendment raised the soil cation exchange capacity (CEC) as compared to the un-inoculated control (Table 4.13). Increase in soil CEC was significantly higher in treatments with biochar addition (BC, BC + *S. indicus* SNW02 and BC + *S. sibiricus* SNW06) as compared to the single mycorrhizal treatments. No significant difference in CEC increment was observed among different treatments with biochar addition.

Table 4.13 Effects of mycorrhizal inoculants and biochar amendment on physico-chemical properties of soil

Treatments		**pH**	**CEC (cmol/kg)**	**TOC (g/kg)**	**Total N (g/kg)**	**Total P (mg/kg)**	**Available P (mg/kg)**
No biochar	Control	6.18±.03ab	14.56±0.9c	12.6±0.3d	1.16±.04b	524±21d	15.73±0.4c
	S. indicus SNW02	6.05±.01cd	15.11±0.7b	14.3±0.6bcd	1.27±.01ab	577±15bc	16.95±0.9abc
	S. sibiricus SNW06	5.96±.02d	15.14±1.2b	14.9±0.4abc	1.32±.02ab	593±12ab	17.14±0.7ab
2% biochar	BC	6.27±.03a	16.32±0.7a	13.8±0.5cd	1.35±.03ab	532±23cd	16.28±0.4bc
	BC+ *S. indicus* SNW02	6.10±.02bc	16.45±1.3a	15.7±0.3ab	1.49±.02a	608±09ab	17.63±0.6a
	BC+ *S. sibiricus* SNW06	5.99±.01cd	16.43±0.6a	16.5±0.7a	1.52±.01a	626±17a	17.92±0.5a

Values (Mean±SD) sharing similar letter within a single column are not significantly different at P<0.05.

Total organic Carbon:

Both mycorrhizal inoculants as well as biochar amendment tended to increase the total organic carbon (TOC) of soils, although not always significantly (Table 4.13). However, increase in TOC was significantly higher when treated with both biochar and mycorrhizal inoculants. Among the dual treatments, increase in TOC was significantly high when treated with BC + *S. sibiricus* SNW06 (16.5 ± 0.7 g/kg) as compared to BC + *S. indicus* SNW02 (15.7 ± 0.3 g/kg). In single mycorrhizal treatments, *S. sibiricus* SNW06 (14.9 ± 0.4 g/kg) treatment showed greater increase in TOC of soils as compared to the *S. indicus* SNW02 (14.3 ± 0.6 g/kg) treatment.

Nutrients:

All the treatments (single mycorrhizal, biochar and biochar plus mycorrhizal) remarkably enhanced the content of total N, total P, and available P in the soil as compared to the control (Table 4.13). The Analysis of Variance (ANOVA) revealed that dual treatments of biochar plus mycorrhizas were more effective than single mycorrhizal treatments in improving nutrients content of the treated soils. It was also observed that no significant difference was found in total N of soils in treatments with anyone of the two mycorrhizal fungi, although the increase in total N was comparatively higher with *S. sibiricus* SNW06. Similarly, total P and available P contents of soil were comparatively higher when treated with *S. sibiricus* SNW06 as compared to *S. indicus* SNW02.

4.4.6 Enzymes activities of Soil

Soil enzymes acts as intermediators and biocatalysts in several biochemical processes, and therefore play crucial role in soil functioning, organic matter decomposition and formation. Soil enzymes also serve as bioindicators of microbial soil activity and substrate availability (Wallenstein et al. 2009), and show quick response to the changes in soil environment (Kandeler et al. 1999; Lagomarsino et al. 2009). In view of this, different enzyme activities such as, acid phosphatase, protease, urease and dehydrogenase were evaluated for the different soil samples collected from the nursery at the end of growing season (Table 4.14).

Acid phosphatase (ACPase) activities in soil samples of all the six treatments were determined in order to study the impacts of mycorrhizal inoculations and biochar amendment on soil ACPase activity. Results showed that both mycorrhizal inoculation as well as biochar amendment tends to increase soil ACPase activities in all the treatments (Table 4.14), with significantly higher values in dual treatment of BC + *S. sibiricus* SNW06 (123 ± 12 µM pNPg^{-1}h^{-1}). Single biochar treatment remarkably enhanced soil ACPase activity to 53 ± 9 µM pNPg^{-1}h^{-1} that was estimated to be 38 ± 5 µM pNPg^{-1}h^{-1} in control (w/o biochar and mycorrhizal fungi). Enhancement in soil ACPase activities was significantly higher in dual treatments of biochar and mycorrhizal fungi as compared to the single mycorrhizal treatments only.

Table 4.14 Effects of mycorrhizal inoculants and biochar amendment on soil enzyme activities

Treatments		Acid phosphatase (µM pNPg^{-1}h^{-1})	Protease (µM NH$_3$g^{-1}h^{-1})	Urease (µM NH$_3$g^{-1}h^{-1})	Dehydrogenase (µg TPFg^{-1}h^{-1})
No biochar	Control	38±5.0d	0.32±0.02e	0.92±0.03e	26.0±2.3d
	S. indicus SNW02	72±4.0c	0.53±0.05cd	1.58±0.14cd	31.0±1.5cd
	S. sibiricus SNW06	96±7.0b	0.68±0.03bc	1.87±0.16c	34.0±0.9bc
2% biochar	BC	53±9.0cd	0.47±0.04de	1.46±0.09d	35.0±2.1bc
	BC+ *S. indicus* SNW02	97±6.0b	0.74±0.09ab	2.24±0.12b	39.0±2.4ab
	BC+ *S. sibiricus* SNW06	123±12.0a	0.89±0.07a	2.62±0.04a	42.0±1.7a

Values (Mean±SD) sharing similar letter within a single column are not significantly different at $P<0.05$.

The behavior of protease and urease enzyme in the samples collected from the nurseries established in present study was also evaluated (Table 4.14). Protease activities were raised in both mycorhizal as well as biochar amended soil treatments, although the increase was significantly higher in dual treatments with mycorrhiza plus biochar. Among the dual treatments, BC + *S. sibiricus* SNW06 treatment enhanced the protease activity greater than BC + *S. indicus* SNW02 treatment. The performance of urease enzyme in the treated soils was quite similar to that of proteases. Urease activities were stimulated by all the treatments, although the increase was variable. Biochar amendment significantly enhanced urease activity in comparison to non-treated control. Similarly, both mycorrhizal inoculations were able to enhance the urease activity significantly as compared to the control. However, the maximum increase in urease activity was found in biochar plus mycorrhizal treatments with highest being in case of BC + *S. sibiricus* SNW06 treatment.

In the present investigation, the biochar treatment as well as single mycorrhizal inoculations remarkably enhanced dehydrogenase activity as compared to the control (Table 4.14). The dehydrogenase activity of microorganisms was detected to be higher when direct mycorrhizal inoculation and biochar amendment were combined. Significantly highest values of dehydrogenase activity were observed with the BC + *S. sibiricus* SNW06 treatment as compared to all other treatments (Table 4.14). The increase observed in the dehydrogenase activity indicates greater microbiological activity as a result of the biochar amendment and mycorrhizal inoculations.

These results revealed that both biochar amendment and mycorrhizal inoculations increased all the enzyme activities but the increase was higher in dual treatments (biochar amendment plus mycorrhizal fungi) than in single mycorrhizal treatments. Further, it was also observed that BC + *S. sibiricus* SNW06 treatment showed significantly higher increase in soil enzyme activities as compared to all other five treatments. Based upon the present investigation, *S. sibiricus* SNW06 was found to be an efficient mycorrhizal strain as compared to other *Suillus* isolates obtained from the northwestern Himalayas and therefore, recommended for the production of mycorrhizal blue pine seedlings in forestry practices. Also biochar application to the soil, especially along with mycorrhizal inoculations, was found to be advantageous for improvement of growth and nutrients content of blue pine seedlings as well as soil physico-chemical properties and enzyme activities. By virtue of this, use of biochar as soil amendment in combination with mycorrhizal inoculants is suggested fruitful and desirable for forestation programmes.

Chapter 5

Discussion

5.1 Diversity and phylogeny of *Suillus* species collected from the northwestern Himalayan region of India

The diversity of the genus *Suillus* was investigated from the northwestern Himalayan region of India during the present research. This study identified seven different *Suillus* species (Table 5.1) from the northwestern Himalayan region of India, based on morpho-anatomical descriptions and molecular analysis of the basidiocarps. Before this study, only two *Suillus* species were reported from this region namely, *Suillus sibiricus* (Sagar and Lakhanpal 2005) and *Suillus granulatus* (Dar et al. 2010). These two species are re-reported and described molecularly for the first time from India. Out of seven species described, 3 taxa *viz. Suillus triacicularis, Suillus indicus* and *Suillus himalayensis* are new to the science and are described both by morphological characters and molecular analysis. *Suillus flavidus* and *Suillus placidus* are two new records of *Suillus* species from this region.

Ecological data regarding abundance and distribution (Table 5.1) suggested that *S. sibiricus* is the most frequently encountered and widely distributed *Suillus* species followed by *S. granulatus* and *S. triacicularis*. *Suillus sibiricus, S. granulatus* and *S. triacicularis* were found distributed throughout the northwestern Himalayan region of India ranging from Jammu and Kashmir (J.&K.) to Uttarakhand (U.K.), whereas only two specimens were recorded each for *S. himalayensis*, *S. indicus*, *S. flavidus* and *S. placidus*. *Suillus himalayensis* and *S. indicus* were observed only from the Barot Valley of Mandi district and Narkanda range of Shimla district of Himachal Pradesh (H.P.), respectively, although probability of their occurrence from other localities can't be denied. *Suillus flavidus* was found only in H.P. as compared to *S. placidus*, which was collected both from H.P. and U.K.

Table 5.1 Ecology and distribution of seven different *Suillus* species identified from the northwestern Himalayas

S. No.	***Suillus* species**	**State of occurrence**	**Voucher No.**	**Host plant**
1	*S. triacicularis* sp. nov.	Jammu and Kashmir, Himachal Pradesh and Uttarakhand	PUN 5523, PUN 5527, PUN 5531, PUN 5534, PUN 5538	PR
2	*S. indicus* sp. nov.	Himachal Pradesh	PUN 6576, PUN 6578	MF (CD & PW)
3	*S. himalayensis* sp. nov.	Himachal Pradesh	PUN 5535, PUN 5537	PW
4	*S. granulatus* (L.) Roussel	Jammu and Kashmir, Himachal Pradesh and Uttarakhand	PUN 5521, PUN 5525, PUN 5526, PUN 5528, PUN 5530, PUN 5533, PUN 5536, PUN 5539, PUN 6573, PUN 6585	PW, CD
5	*S. sibiricus* (Singer) Singer	Jammu and Kashmir, Himachal Pradesh and Uttarakhand	PUN 5520, PUN 5522, PUN 5524, PUN 5529, PUN 5532, PUN 6569, PUN 6570, PUN 6572, PUN 6575, PUN 6577, PUN 6579, PUN 6581, PUN 6582, PUN 6583, PUN 6586, PUN 6587, PUN 6588	PW, CD, PS
6	*S. flavidus* (Fr.) Singer	Himachal Pradesh	PUN 6574, PUN 6580	PW
7	*S. placidus* (Bonord.) Singer	Himachal Pradesh and Uttarakhand	PUN 6571, PUN 6584	PW

*PR, PW, CD, PS, and MF stands for *Pinus roxburghii*, *Pinus wallichiana*, *Cedrus deodara*, *Picea smithiana* and mixed forest type, respectively.

The field experience suggested that *Suillus* species are mainly associated with the members of family *Pinaceae* (Table 5.1). Most of them were found associated with *Pinus wallichiana*, but some also with *Pinus roxburghii*, *Cedrus deodara* and *Picea smithiana* trees. *Suillus sibiricus* and *S. granulatus* were found mainly with *P. wallichiana* and rarely with *C. deodara*. In addition, one specimen of *S. sibiricus* (SUK20) was also found with *Picea smithiana*. *Suillus himalayensis*, *S. flavidus* and *S.*

placidus were found only in pure *P. wallichiana* forest, whereas *S. indicus* specimens were found from the mixed forests of *C. deodara* and *P. wallichiana*. *Suillus triacicularis* was found fruiting exclusively in association with *P. roxburghii* trees. The comparative characteristics of all the *Suillus* species recorded from the northwestern Himalayas are further discussed as follows:

Suillus triacicularis morphologically resembles to *Suillus granulatus* (L.) Roussel and *Suillus collinitus* (Fr.) Kuntze. The absence of watery green context above the tubes during any stage of development distinguishes it from *S. granulatus*. The species looks very similar to pallid variants of *S. granulatus* when young, but differs by the absence of cloudy droplets over the tubes. Moreover, the yellow to reddish or orange-yellow pileus at maturity separates it from *S. granulatus*, which has a cinnamon brown pileus at maturity. *Suillus collinitus*, which is very similar to *S. granulatus*, can be distinguished by its pinkish mycelia at the stipe base as compared to pure white mycelia in *S. granulatus* and *S. triacicularis*. The closest ITS sequence of *S. triacicularis* found in GenBank was *S. collinitus* isolate CCMA-79 (AY935517) with 95% similarity (99% coverage) followed by *S. collinitus* isolate CCMA-46 (AY935516) with 95% similarity (99% coverage). The phylogenetic analysis of ITS region (Fig. 4.17) also formed a well-supported clade of *S. triacicularis*, which differed from other previously reported species of the genus *Suillus*. Based on morphological features and molecular characterization, *S. triacicularis* was designated as a new species.

Suillus indicus is close to *Suillus decipiens* (Peck) Kuntze but differs considerably identifying it as a separate species. The presence of umbo, less numerous/prominent fibrillose squamules over the pileal surface and absence of squamules over the stipe differentiate it from *S. decipiens*. Anatomically, the occasional presence of 2-spored basidia and complete absence of caulocystidia distinguish the species from *S. decipiens*. *Suillus spraguei* (Berk. and Curt.) Kuntze is the next closest species, which is commonly referred to as *Suillus pictus* A.H. Sm. and Thiers although the name *S. spraguei* is used for one or even several disjunct species of *S. pictus* in Asia (Wu et al. 2000; Burchhardt et al. 2011; Klofac 2013). When fresh, the pileus of *S. spraguei* fresh specimens is much redder than *S. decipiens* and *S. indicus* but the faded specimens strongly resemble *S. decipiens*. In contrast to *S. indicus*, *S. spraguei* also bear fibrillose squamules on the stipe. The taxonomic distinctness of *S. indicus* from other species is further confirmed by the molecular analysis. The closest ITS sequence of *S. indicus* found in GenBank was *S. flavidus* isolate FFP962 (JQ711908) with 93% similarity (99% coverage) followed by *S. flavidus* isolate FFP886 (JQ711885) with 93% similarity (99% coverage). The phylogenetic inference based on ITS sequences (Fig. 4.17) clearly separated the present specimen from all other species of *Suillus* reported earlier and describe it as a new species.

Morphologically, *Suillus himalayensis* come close to *Suillus americanus* (Peck) Snell and *Suillus sibiricus* (Singer) Singer. Till date, there is no record for *S. americanus* from India and *S. sibiricus* is the abundantly fruiting and most dominating *Suillus* species associated with *P. wallichiana* in the northwestern Himalayas. *Suillus americanus* is the nearest relative of *S. himalayensis*. The species varies in possessing the yellow white to pale yellow pileus with light greenish tinge, brownish squamules, white pileal veil, upturned margin and plane apex as compared to the bright yellow pileus surface, orange to reddish squamules, yellowish pileal veil, incurved margin and occasional presence of low obtuse umbo in *S. americanus.* Also, the species differs as its flesh changes to grayish green and tubes to olive brown color on bruising in contrast to vinaceous brown in *S. americanus*. Microscopically, the less broaden cystidial elements (3.8–6.2 µm) than *S. americanus* (7–12 µm) is the main distinguishing characteristic of the present specimens. Basidia are larger and basidiospores more cylindrical (Q=3.0) than *S. americanus* (Q=2.5). The second nearest species is *S. sibiricus*, but it differs from that of *S. himalayensis* by the pileus rather dingy olive yellow, flesh and tubes changing to dull cinnamon on bruising, tubes 5–15 mm, slight umbo present occasionally, and the basidiospores being less cylindrical (Q=2.3). When compared with GenBank database, ITS sequences of *S. himalayensis* showed closest similarity with *S. sibiricus* strain HMAS-66061 (AF166512) with 99% similarity (98% coverage) followed by *S. flavidus* isolate FFP962 (JQ711908) with 98% similarity (99% coverage). The Bayesian analysis of ITS region (Fig. 4.17) shows that ITS sequences of *S. himalayensis* form a separate clade. The presence of distinctive taxonomic features and a well-supported phylogenetic clade supports the dissimilarity of the described species with respect to other species of *Suillus* and therefore erected as a new species.

Suillus granulatus sporocarps collected from the northwestern Himalayas resemble well with *S. granulatus* described by Smith and thiers (1964) and Corner (1972). However, they differ by having few larger basidia up to 30 µm in length. Smith and Thiers (1964) found it from *Pinus strobus* forest and Corner (1972) recorded it from mixed conifer forest growing under white pines. The present specimens were collected mostly from pure *P. wallichiana* forests and rarely from *C. deodara* forests. Yellowish brown to light brown and cinnamon brown pileus and unchanging pileal context distinguish it from *S. placidus* (Bonord.) Singer, which has white to pale yellow pileus and vinaceous staining pileal context. The nearest ITS sequence of *S. granulatus* found in GenBank was *S. granulatus* isolate S070 (AJ272410) with 99% similarity (98%

coverage) and next closet sequence was *S. granulatus* isolate AT259 (AJ272408) with 99% similarity (98% coverage). The phylogenetic analysis clustered the ITS sequences of *S. granulatus* derived in the present study with the ITS sequences of Asian *S. granulatus* (L54121) and European *S. granulatus* (AJ272408, AJ272410). Thus, morphological as well as phylogenetic analysis confirms the present specimens as *S. granulatus*.

The specimens of *S. sibiricus* collected from northwestern Himalayas closely resemble to *S. sibiricus* (Singer) Singer (Smith and Thiers 1964). Morphologically, the sporocarps are very similar to *S. himalayensis* (Verma and Reddy) and *S. flavidus* (Fr.) Singer. It differs from that of *S. himalayensis* by the pileus rather dingy olive yellow, flesh and tubes changing to dull cinnamon on bruising, tubes 5–15 mm, slight umbo present occasionally, and the basidiospores being less cylindrical (Q=2.3). The second closest species is *S. flavidus*, but can be distinguished by the pileal surface that is floccose to squamulose in *S. sibiricus* and glabrous in *S. flavidus*. Smith and Thiers (1964) observed it growing gregarious under *Pinus monticola*. The populations from northwestern Himalayas have been recorded mainly from *P. wallichiana* forests. Occasional specimens have been found in association with *C. deodara* and *Picea smithiana*. The closest ITS sequence of *S. sibiricus* found in GenBank was *S. sibiricus* strain HMAS-66061 (AF166512) with 99% similarity (98% coverage), which was followed by *S. flavidus* isolate FFP962 (JQ711908) with 98% similarity (99% coverage). Further, the ITS sequences of *S. sibiricus* specimens derived in the present study are clustered along with the ITS sequences of *S. sibiricus* derived from different parts of the world (Fig. 4.17). As a result of morphological and molecular identification, the present specimens are confirmed as *S. sibiricus*.

The sporocarps of *Suillus flavidus* recorded from northwestern Himalayas resembles well with those described from Pakistan by Sarwar et al. (2012) in association with *P. wallichiana*. The species is characterized by its yellow to yellowish brown glabrous pileus with patches of gluten, pallid to pale yellowish glandular dots on the stipe and a whitish thick annulus staining brownish on handling. Morphologically the species is close to *S. sibiricus,* but can be differentiated by its glabrous pileal surface as compared to floccose or squamulose in *S. sibiricus*. The ITS sequence of present specimen showed closest similarity with *S. flavidus* isolate FFP962 (JQ711908) with 99% similarity (99% coverage) and the next closet sequence was *S. flavidus* voucher SMI206 (FJ845439) with 99% similarity (99% coverage). Further, the phylogenetic analysis based on ITS region (Fig. 4.17) clustered the ITS sequence in a clade comprising the ITS sequences of *S. flavidus* already available in Genbank database. This data identified the present specimens as *S. flavidus*, which is a new record from India.

The specimens of *Suillus placidus* collected from the northwestern Himalayas closely resemble to those described by Smith and thiers (1964) and Corner (1972). They found it growing gregarious under *P. strobus* trees whereas, the present specimens were collected in association with *P. wallichiana* trees. It is characterized by pure white pileus when young, becoming pale yellow with age and vinaceous brown glandular dots or smears throughout the stipe surface. It is close to *S. granulatus* (L.) Roussel, but can be distinguished by white to pale yellow pileus and vinaceous staining of pileal context on bruising. On the other hand, *S. granulatus* have yellowish brown to light brown and cinnamon brown pileus and unchanging pileal context. The closest ITS sequence of *S. placidus* found in GenBank was *S. placidus* isolate Chu (DQ407265) with 98% similarity (99% coverage). The following another closet sequence was *S. placidus* isolate VC-1022 (L54118) with 99% similarity (92% coverage). The phylogenetic analysis based up on ITS region (Fig. 4.17) also supported the specimen as *S. placidus*, which is another new record from India found in the present study.

Bayesian analysis of ITS region of selected *Suillus* species yielded a consensus tree (Fig. 4.17), which revealed that *Suillus* species are clustered into two different groups, *viz. Suillus* group I and *Suillus* group II. According to Smith and Thiers (1964) and Klofac (2013), these groups can be morphologically described on the basis of a few leading morphological characteristics of the *Suillus* species such as, presence or absence of veil and/or annulus on the basidiocarps. All the species in *Suillus* group I, except *S. placidus* (Bonord.) Singer, comprises mainly of the species possessing a well developed veil or false veil in young specimens either leaving an annular zone or true annulus on the stipe, or otherwise adhered to the pileal margin at maturity. Contrastingly, the *Suillus* group II comprises of the species in which either only annulus is absent or otherwise both annulus and veil are absent on the basidiocarps. Despite the fact that *S. placidus* is phylogenetically related to *Suillus* group I, morphologically it resembles *Suillus* group II due to absence of veil and annulus on basidiocarps during any stage of development (Singer 1945; Smith and Thiers 1964). Thus, the *Suillus* species under consideration are phylogenetically grouped on the basis of their leading morphological features with few exceptions. These results are in concurrence to Mleczko and Ronikier (2007), who have proposed that classical basidiocarp-based characteristics in the genus *Suillus* are not in much congruence with phylogenetic relationships (Mleczko and Ronikier (2007).

In conclusion, the present work enhanced our knowledge about diversity, ecology and phylogeny of *Suillus* species occurring in the northwestern Himalayan region of India. Further, 21 ITS sequences of Indian *Suillus* species have been deposited in the GenBank database, which would be useful for comparing the sequences of unidentified specimens. In addition, the deposition of ITS sequences had provided a phylogenetic framework for biogeographical analysis of *Suillus* species.

5.2 Physiological characterization and *in vitro* evaluation of *Suillus* isolates

The ability of ECM fungi to grow fast under *in vitro* conditions is an important criterion for selection of ECM fungi for mass cultivation (Trappe 1977; Brundrett et al. 1996; Beatriz et al. 2006). Significant inter-specific differences were found in radial growth and mycelia dry weight of *Suillus* isolates when grown under *in vitro* conditions (Fig. 4.19 & Fig. 4.20). 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. Evaluation of *in vitro* fungal growth was the first step toward selection of efficient *Suillus* isolates for mass inoculum production. Furthermore, *in vitro* mycorrhizal root colonization of *P. wallichiana* roots by *Suillus* isolates was studied. The results revealed that *Suillus* species differ remarkably in their ability to colonize *P. wallichiana* roots (Fig. 4.26). Variations in the root colonization were also observed even within the isolates of *S. sibiricus* (isolate SNW05–SNW08). 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. In addition, different extracellular enzyme activities of ECM fungi primarily involved in nitrogen and phosphorus uptake were determined for all the *Suillus* isolates. It was observed that all the *Suillus* isolates showed acid phosphatase, phytase, protease and chitinase activities when grown *in vitro* (Table 4.6). Significant inter-specific as well as intra-specific differences were detected within the extracellular enzyme activities of different *Suillus* isolates. Numerous studies have reported acid phosphatase (Raman et al. 1998, 2002; Tibbett et al. 1998, Wannet et al. 2000; Jayakumar and Tan 2005; Quiquampoix and Mousain 2005; Alvarez et al. 2006; Baghel et al. 2009; Nygren and Rosling 2009; Louche et al. 2010; Bechem 2013), phytase (Quiquampoix and Mousain 2005), protease (Maijala et al. 1991; Leake 1996; Tibbett et al. 1999; Nehls et al. 2001) and chitinase (Leake and Read 1990b; Lindahl and Taylor 2004) activities from a number of ECM fungi.

To select the efficient *Suillus* isolates for mass inoculum production and field inoculations, *in vitro* growth and nutrients content of ectomycorrhizal blue pine (*P. wallichiana*) seedlings were studied. Outcomes of the present investigation (Table 4.7 & Table 4.8) suggested that all the *Suillus* isolates tended to increase seedlings growth (seedlings height, root length, fresh weight and dry weight) and nutrient uptake (N, P, K, Mg and Ca) as compared to the un-inoculated control seedlings. Following ectomycorrhizal (ECM) 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 (Mejstřík 1975; Cumming 1993; Guerin-Laguette et al. 2004; Rincón et al. 2005a; Sim and Eom 2006; Beatriz et al. 2006; Dalong et al. 2011; Sousa et al. 2012). Moreover, the ECM inoculation of blue pine seedlings with different ECM fungi have been also shown to increase growth, biomass, and nutrient contents of the ectomycorrhizal blue pine seedlings (Dar et al. 2007, 2010; Ahangar et al. 2012; Itoo and Reshi 2014a).

In the present study, *S. sibiricus* isolate SNW06 showed significantly higher plant growth and biomass in comparison to other *Suillus* isolates (Table 4.7). 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. While contemplating *in vitro* mycorrhizal capacity of different indigenous *Suillus* isolates for the growth of *Pinus halepensis* seedlings (Beatriz et al. 2006), it was noticed that *Suillus* isolates stimulated the growth of *P. halepensis* seedlings in different rates.

Further, all the *Suillus* isolates increased the content of nutrients in blue pine seedlings and this increase in nutrients content was variable with different isolates (Table 4.8). The overall increase in nutrients content was significantly higher in treatment with *S. sibiricus* SNW06, which was followed by *S. indicus* SNW02 and *S. sibiricus* SNW07, respectively. In contrast, *S. himalayensis* SNW03 showed significantly lowest increase in total nutrients content of blue pine seedlings. Dalong et al. (2011) evaluated the growth response and nutrient uptake of *P. densiflora* seedlings when inoculated with three indigenous ECM fungi (*Cenococcum geophilum*, *Rhizopogon roseolus* and *Russula densifolia*) and found that the content of nutrients in seedlings was altered to varying extent, depending on the fungus and the nutrient considered. Similarly, Growth response and nutrient uptake of *P. wallichiana* seedlings when inoculated with three different microbial inoculants (*Trichoderma harzianum, Pseudomonas fluorescens, Laccaria laccata*) was studied by Ahangar et al. (2012) and observed that uptake of nutrients (N, P, K) in *P. wallichiana* seedlings was significantly improved by microbial inoculants, tested individually or in combination.

All these results revealed that *Suillus* species exhibit considerable inter-specific as well as intra-specific variations in their physiological traits, such as growth rates, extracellular enzyme activities, plant root colonization ability and mycorrhizal capacity. Based on these differentiating attributes, suitable and efficient *Suillus* isolates were selected for mass inoculum production with an aim of blue pine afforestation. The present study identified *S. sibiricus* SNW06 and *S. indicus* SNW02 as efficient *Suillus* isolates for enhancement of growth, biomass and nutrients content of blue pine seedlings and therefore selected for mass inoculum production and field trials.

5.3 Optimization of culture conditions for mass inoculum production

The pine species employed in reforestation programs mainly rely upon ECM fungi for their early establishment, survival and growth (Smith and Read 1997). The utilization of ECM fungi to improve forest productivity requires scale-up the cultivation of fungal inoculants. To produce the mass inoculum, it is essential to define the optimal medium composition and culture conditions for each ECM fungus. In this study, different medium composition and culture conditions were optimized with an aim to enhance the growth of selected *Suillus* isolates (*S. indicus* SNW02 and *S. sibiricus* SNW06) for mass inoculum production. In a preliminary set of experiments, the radial growth of both the *Suillus* isolates was tested on three different basic fungal media (Table 4.9). Growth of both the isolates was found maximum on 2% Malt Extract (ME) agar medium as compared to Modified Melin-Norkrans (MMN) agar medium and Potato dextrose agar (PDA) medium. In general, the ECM fungi exhibit different growth on different culture media (Radhakrishnan and Mohan 2008; Rossi and Oliveira 2011; Islam and Ohga 2013). Further, the concentration of ME was varied in medium for both the isolates and it was observed that *S. indicus* SNW02 showed maximum growth at 5 g/l ME concentration and *S. sibiricus* SNW06 showed maximum growth at 2.5 g/l ME concentration (Fig. 4.27).

We also examined the effects of nine different carbohydrate sources and five different nitrogen sources on the radial growth of both the *Suillus* isolates (Fig. 4.28 & Fig. 4.30). The results revealed that utilization of carbohydrate sources differed considerably between *S. indicus* SNW02 and *S. sibiricus* SNW06 (Fig. 4.28). Remarkable differences in utilization of carbohydrate sources among various strains of *Boletus* and *Suillus* have also been reported earlier by various authors (Ferry and Das 1968; Lamb 1974; Murata 1993; Hatakeyama and Ohmasa 2004). Besides, present outcomes are also supported by the observations of Itoo and Reshi (2014b) who found differences in the utilization of the different carbon sources between six different ECM fungi. *Suillus indicus* SNW02 grew well on most of the carbohydrate sources, except sorbitol and galactose. Among the different carbohydrate sources tested, the growth of *S. indicus* SNW02 was higher and equally well in ME medium, supplemented either with glucose or sucrose followed by fructose. On the other hand, *S. sibiricus* SNW06 showed good growth in all of the carbohydrate sources tested including sorbitol and galactose. Thus, the utilization of sorbitol and galactose differed considerably among both the *Suillus* isolates. The radial growth of *S. sibiricus* SNW06 was higher in media containing galactose, glucose or sucrose as carbon sources and the maximum growth was obtained when supplemented with glucose. These finding suggested that glucose can be used as an optimum carbon source for mass inoculum production of both the *Suillus* isolates. While working with different ECM fungi, several other authors (Daza et al. 2006; Guler and Özkaya 2008; Akata et al. 2012) have also reported glucose as one of the optimal carbon sources for mycelial growth of ECM fungi. Further, the glucose was applied to the respective media at varying concentrations and radial growth of both the isolates was evaluated (Fig. 4.29). Although the maximal radial growth for *Suillus indicus* SNW02 was found at 20 g/l of glucose concentration and for *S. sibiricus* SNW06 at 15 g/l of glucose concentration, both the *Suillus* isolates were capable of growing well even at very high concentration of glucose (30 g/l). All the *Suillus* strains studied by Hatakeyama and Ohmasa (2004) also grew well at a glucose concentration of 33.3 g/l and one of the *Suillus* strain (*S. luteus* SA50) was found to exhibit best growth at 100 g/l of glucose concentration. Moreover, *Pisolithus microcarpus* have been shown to have maximum biomass yield, when grown in MMN medium containing 40 g/l glucose concentration (Rossi and Oliveira 2011). Itoo and Reshi (2014b) tested six ECM fungi and found that all the species grew well at a glucose concentration of 20 g/l. These results reveal that ECM fungal species can grow well at comparatively higher glucose concentrations in the medium. This may be attributed to the fact that ECM fungi often met high sugar concentration micro-environment in the root system of the plants (Hatakeyama and Ohmasa 2004).

As described in the results (Fig. 4.30), the growth of both the *Suillus* isolates varied in response to all the five nitrogen sources tested. All the nitrogen sources allowed good growth, but the growth was generally higher in medium with inorganic nitrogen sources (di-ammonium hydrogen phosphate and potassium nitrate) than with organic nitrogen sources (alanine, arginine and ammonium tartrate). Similar results have been reported by other authors (Sarjala 1999; Itoo and Reshi 2014b) while investigating the growth of different ECM fungi grown in media supplemented with different inorganic and organic nitrogen sources. In addition, both the *Suillus* isolates utilized nitrogen better in ammonical form (di-ammonium hydrogen phosphate) as compared to the nitrate form (potassium nitrate). Ammonium form of nitrogen has also been identified as most readily utilizable source of nitrogen for a number of ECM fungi (Rangel-Castro et al. 2002; Sangtiean and Schmidt 2002; Itoo and Reshi 2014b). Further support is drawn from the several other observations (Nygren et al. 2008; Sawyer et al. 2003; Daza et al. 2006; Itoo and Reshi 2014b), which illustrated that growth of ECM fungi is low in medium containing nitrate as nitrogen source. As di-ammonium hydrogen phosphate [$(NH_4)_2HPO_4$)] was found optimum nitrogen source for growth enhancement of both the *Suillus* isolates, concentration of $(NH_4)_2HPO_4$ in medium was further optimized by keeping the glucose concentration constant (Fig. 4.31). Both the *Suillus* species showed an increase in radial growth with increasing concentration of $(NH_4)_2HPO_4$ in the media. The maximum growth was achieved at a specific level of nitrogen source (10 g/l $(NH_4)_2HPO_4$ concentration for *S. indicus* SNW02 and 5 g/l $(NH_4)_2HPO_4$ concentration for *S. sibiricus* SNW06) and growth retarded thereafter. This shows that keeping the carbon concentration constant; nitrogen concentration is the limiting factor for growth of these isolates. Thus, C/N ratio is crucial for the growth of these *Suillus* isolates. The C/N ratio of growth medium has been considered as key factor in determining fungal growth (Chang and Miles 1989; Wallander et al. 2003; Fransson et al. 2007).

As observed by Zhang et al. (2010) for strains of *S. luteus*, adenosine tends to promote the radial growth of both the *Suillus* isolates tested in this study (Fig. 4.32). The radial growth of these isolates increased up to a certain concentration of adenosine in media and then started decreasing. *Suillus indicus* SNW02 showed maximum growth at 0.16 g/l adenosine concentration and *S. sibiricus* SNW06 showed maximum growth at 0.08 g/l adenosine concentration in the medium. Higher concentrations of adenosine (≥0.32 g/l) in medium inhibited the radial growth of both the *Suillus* isolates. Almost similar results were obtained by Zhang et al. (2010), while working with three different strains of *S. luteus, viz. S. luteus* SNP-20, *S. luteus* SAF-501 and *S. luteus* PT-501.

Finally, temperature and pH were optimized for growth of both the *Suillus* isolates. Growth parameters, such as temperature and pH, greatly influence the growth of ECM fungi in the field as well as under controlled conditions in the laboratory. Numerous authors (Hacskaylo et al. 1965; Hung and Trappe 1983; Dennis 1985; Sánchez et al. 2001; Daza et al. 2006; Xu et al. 2008) have examined the influence of temperature and pH on the growth of ECM fungi. In this study (Fig. 4.33), the maximum radial growth for *S. indicus* SNW02 was observed at 25°C and for *S. sibiricus* SNW06 at 30°C. *Suillus indicus* SNW02 was incapable of growing at 40°C, whereas *S. sibiricus* SNW06 showed much retarded growth at 40°C. Dennis (1985) analyzed 67 isolates of ECM fungi and found that optimum temperature for the growth of ECM fungi ranges from 15–30°C with 45 isolates growing best at 25°C. Further support in favor of our study is drawn from the findings of Daza et al. (2006) and Xu et al. (2008), who observed optimum temperature ranging from 24–28°C for the ECM fungi tested. Xu et al. (2008) also revealed that all the three isolates (*Lactarius deliciosus*, *Boletus edulis* and *Lactarius insulsus*) of ECM fungi tested showed no growth at 5°C and 40°C, and suggested that even a small decrease or increase from optimum temperature has a negative impact on the fungal growth.

Although maximum growth was observed in acidic pH for both the isolates, but the optimum pH varied considerably (Fig. 4.34). *Suillus indicus* SNW02 showed maximum radial growth at pH 5.0, whereas *S. sibiricus* SNW06 showed maximum radial growth at pH 6.0. The optimum pH for growth of 67 isolates of ECM fungi was found to range from 4–7 and none of the ECM fungi grew at a pH greater than 9 (Dennis 1985). Several other investigations (Sánchez et al. 2001; Daza et al. 2006; Xu et al. 2008) have also illustrated that the pH optima for maximal growth of ECM fungi differ considerably among different species or strains.

At the end, optimization of all these parameters have led to development of two optimized media i.e. optimized medium 1 (OM1) for *S. indicus* SNW02 and optimized medium 2 (OM2) for *S. sibiricus* SNW06. For *S. indicus* SNW02, 1.58 fold increase was obtained in radial growth when grown in OM1 than the initial growth in 2% ME medium. In case of *S. sibiricus* SNW06, 1.54 fold increase was achieved in radial growth when grown in OM2 as compared to the initial growth in 2% ME medium. Moreover, the biomass yield increased 1.46 fold in *S. indicus* SNW02 and 1.39 fold in *S. sibiricus* SNW06 when grown in respective optimized media as compared to the initial yield in 2% ME medium. Thus, the optimization of culture conditions have remarkably increased the radial growth as well as the biomass yield of both the selected *Suillus* isolates, which is crucial for mass inoculum production in forestry practices.

5.4 Influence of selected *Suillus* species and biochar amendment on plant growth and rhizosphere properties

As per the outlined objectives, the effects of selected *Suillus* species (*S. indicus* SNW02 and *S. sibiricus* SNW06) and biochar amendment on plant growth and rhizosphere properties were studied after conducting a nursery trial under open field conditions. As discussed earlier for *in vitro* evaluation of *Suillus* isolates, mycorrhizal inoculations of blue pine (*P. wallichiana*) seedlings with both the *Suillus* isolates increased root colonization of pine roots, seedlings growth, biomass and nutrients content than the un-inoculated control seedlings (Table 4.11 & Table 4.12). When compared to each other, inoculation with *S. sibiricus* SNW06 showed better root colonization, seedlings growth, biomass, and nutrients uptake than with *S. indicus* SNW02.

The results further revealed that mycorrhizal inoculations with both *Suillus* isolates altered the rhizospheric properties of the soil (Table 4.13). The pH of the soil was decreased significantly and the soil cation exchange capacity (CEC) was increased significantly in mycorrhizal treatments as compared to the non mycorrhizal treatment. Decrease in soil pH due to mycorrhizal inoculations has also been observed by other authors (Garcia et al. 2000; Liu et al. 2008). Liu et al. (2008) have suggested that mycorrhizal plants with high ECM tip density take up nitrogen (N) in the ammonium (NH_4^+) form rather than in nitrate (NO_3^-) form and the mycorrhizal plants excrete hydrogen ion (H^+) to the soil to maintain the electro-neutrality within the plant. As a result, pH of rhizosphere is expected to decrease. A slight increase in soil electrical conductivity (EC) due to direct mycorrhizal treatment and a significant increase due to indirect mycorrhizal treatment have also been detected earlier by Garcia et al. (2000).

Inoculation with both *Suillus* isolates (SNW02 and SNW06) tended to increase the total organic carbon (TOC) content of the soil. This may be attributed to the increased plant growth attained with mycorrhizal treatments as compared to the control, suggesting an enhanced C allocation to the soil (Roldán et al. 1996; Garcia et al. 2000). Furthermore, the content of nutrients (total N, total P, and available P) in the soils was increased when treated with any of the *Suillus* isolates as compared to the control. This is in agreement with other authors, who also found an increase in nutrients content of the soil when treated with mycorrhizal fungi (Garcia et al. 2000; Caravaca et al. 2002; Dar et al. 2007; Liu et al. 2008). Mycorrhizal fungi are suggested to bring about important mineral transformation in soil and allow access to unavailable forms of nutrients by breaking down the organic macromolecules into simple accessible forms, which may further led to an increase in the availability of certain metals in the soil (Cairney and Chambers, 1997). For example, mycorrhizal fungi can mineralize organic phosphorus (P) reservoir of soils into plant accessible inorganic orthophosphate (Pi) (Smith and Read 1997; Querejeta et al., 1998) that enhance the content of available P in the soils. In this study, enhancement in the soil nutrients was observed due to mycorrhizal inoculation with both the *Suillus* isolates and comparatively better results were obtained in treatment with *S. sibiricus* SNW06 than with *S. indicus* SNW02. Inoculation with both the *Suillus* isolates also increased the soil enzyme activities such as, acid phosphatase, protease, urease and dehydrogenase. The increased enzyme activities in the rhizosphere of mycorrhizal plants have also been reported earlier by many authors (Garcia et al. 2000; Caravaca et al. 2002; Vázquez et al. 2000; Liu et al. 2008).

While evaluating the influence of biochar on mycorrhizal root colonization, it was observed that biochar application slightly increased mycorrhizal root colonization as compared to the non-biochar treatments (Table 4.11). Both, BC + *S. indicus* SNW02 and BC + *S. sibiricus* SNW06 treatment showed increased mycorrhizal root colonization as compared to single mycorrhizal treatments, but no significant differences was found in root colonization percentages. BC+ *S. sibiricus* SNW06 treatment showed the highest root colonization as compared to all other treatments. Numerous studies have reported an increased in mycorrhizal root colonization due to biochar addition (Harvey et al. 1976; Saito 1990; Ishii and Kodoya 1994; Mori and Marjenah 1994; Ezawa et al. 2002; Matsubara et al. 2002; Yamato et al. 2006; Rillig et al. 2010). Moreover, higher root colonization (Harvey et al. 1976) and earlier onset of mycorrhizal formation (Herrmann et al. 2004) have been reported earlier with low concentrations (< 2%) of activated charcoal. Treatment of sub-boreal forest soil with 5 and 10% biochar increased only the abundance of *Rhizopogon-Suillus* sp. (only in fertilized soil) and *Cenococcum* (in fertilized and non-fertilized soil), and did not influenced the abundance of most of the other ECM morphotypes (Robertson et al. 2012). On the other hand, a considerable decrease in ECM colonization of *Pinus densiflora* and *Larix kaempferi* roots has been observed by Choi et al. (2009) when the forest soil was amended with 30% biochar. These results demonstrate that there is an upper limit beyond which biochar addition may negatively affect the formation or abundance of ECMs in soil.

As a result of increased mycorrhizal root colonization, biochar addition supplemented with the *Suillus* inoculants tended to improve the growth and biomass of blue pine seedlings (Table 4.11). In the present study, BC + *S. sibiricus* SNW06 treated seedlings showed higher enhancement in plant height, root length, shoot biomass and root biomass of the seedlings as compared to BC + *S. indicus* SNW02 treated seedlings. Single biochar treatment without any mycorrhizal fungus also improved the growth and biomass of blue pine seedlings. Several authors have reported the positive effects of biochar on plant growth and biomass as a result of biochar additions (Iswaran et al. 1980; Chidumayo 1994; Wardle et al. 1998; Hoshi 2001; Lehmann et al. 2002; Chan et al. 2007; Van Zwieten et al. 2007; Kimetu et al. 2008; Robertson et al. 2012). Glaser et al. (2001) have illustrated that low biochar additions (0.5 t/ha) generally tended to have positive impacts on various crop species, whereas higher biochar rates inhibits crop biomass and/or productivity. In addition, biochar application to the soil stimulated the nutrients uptake by blue pine seedlings (Table 4.12). Among all biochar treatments, dual treatment of biochar and mycorrhizal fungi showed significantly higher nutrients content in blue pine seedlings. In dual treatments, BC + *S. sibiricus* SNW06 treatment showed significantly higher nutrients content in seedlings than BC + *S. indicus* SNW02 treatment. This increase in nutrients content of seedlings may be attributed to enhanced root colonization (Yamato et al. 2006; Rillig et al. 2010) and improved soil physicochemical properties (Lehmann and Rondon 2006; Fowles 2007; Rondon et al. 2007; Blackwell et al. 2009; Downie et al. 2009) due to biochar additions.

Effect of biochar amendment on soil properties was also evaluated (Table 4.13). Biochar addition slightly increased the soil pH that may be due to alkaline nature (pH 7.8) of the pine needle biochar used in this study. As found in case of mycorrhizal inoculation, biochar amendment also increased the soil cation exchange capacity (CEC) as compared to the un-inoculated control. Increase in soil CEC was significantly higher in treatments with biochar addition (BC, BC + *S. indicus* SNW02 and BC + *S. sibiricus*

SNW06) as compared to non-biochar treatments. Similar increase in soil CEC as a result of biochar amendment has been recorded earlier by several authors (Chan et al. 2008; Van Zwieten et al. 2010; Nigussie et al. 2012; Agusalim et al. 2010; Robertson et al. 2012). As anticipated, biochar addition increased TOC of the soils. Basically, biochar mainly consists of stable aromatic forms of organic carbon that leads to an increase in the recalcitrant C fraction of the soil. Further, the biochar amendment also increased the pine growth with respect to the same treatments without biochar that in turn led to an enhanced C allocation to the soil. Further down the study, all the biochar treatments significantly enhanced the content of total N, total P, and available P in the soil as compared to non-biochar treatments. Tendency of biochar to increase the soil cation exchange capacity (Van Zwieten et al. 2010; Nigussie et al. 2012; Robertson et al. 2012; Glaser et al. 2002) and alter the soil physicochemical properties (Lehmann and Rondon 2006; Fowles 2007; Rondon et al. 2007; Blackwell et al. 2009; Chan and Xu 2009; Downie et al. 2009) is suggested to increase the availability of nutrients in soils (Fowles 2007; Warnock et al. 2007). Also, the soil nutrients can bind to the biochar that prevents the subsequent nutrient run-off from the soil (Fowles 2007). In fact, the collective properties of biochar to increase soil pH (Glaser et al. 2002; Matsubara et al. 2002; Yamato et al. 2006; Rondon et al. 2007; Van Zwieten et al. 2007), increase soil CEC (Van Zwieten et al. 2010; Nigussie et al. 2012; Robertson et al. 2012), improve soil physicochemical properties (Rondon et al. 2007; Blackwell et al. 2009; Chan and Xu 2009; Downie et al. 2009), and retaining soil nutrients (Hoshi 2001; Lehmann et al. 2002, 2003a; Lehmann 2007) are the possible contributing factors responsible for increase in plant productivity.

Biochar amendment also increased all the enzyme activities tested in biochar treated soils and the increase was highest in dual treatment of BC + *S. sibiricus* SNW06 (Table 4.14). It has been suggested that biochar provide favorable growth conditions (Fowles 2007) and protection from predators (Ezawa et al. 2002; Samonin and Elikova 2004) to the soil micro-biota. Thus, biochar serves as a refuge for soil microorganism colonizing the biochar amended soil. In addition, the organic substrates and nutrients bound to the biochar provide nourishment to the soil microorganisms (Hamer et al. 2004; Atkinson et al. 2010). As a result, the microbial biomass and activities of biochar amended soils get increased. The protection and nutrients provided by biochar may also led to an increase in abundance of mycorrhization helper bacteria (MHBs), phosphate solubilizing bacteria (PSBs) and root colonizing mycorrhizal fungi (Warnock et al. 2007), which further results in increased microbial activities.

In conclusion, both mycorrhizal inoculants and biochar amendment used in present study stimulated the growth, biomass and nutrients content of blue pine seedlings and the enhancement was better when the seedlings were treated with a combination of mycorrhizal fungus and biochar. Biochar treatment also increased the ECM colonization of blue pine roots as compared to non-biochar treatment. In addition, the soil physico-chemical properties were also altered by mycorrhizal inoculants and biochar amendment and more profound results were obtained when soils were treated both with mycorrhizal fungi and biochar additions. Further, the dual treatment of mycorrhizal fungi plus biochar amendment also showed significantly higher increase in soil enzyme activities as compared to the other treatments. On the basis of these findings, BC + *S. sibiricus* SNW06 treatment was found most advantageous for improvement of growth, biomass and nutrients content of blue pine seedlings as well as soil physico-chemical properties and enzyme activities.

Chapter 6

Summary

Diversity of *Suillus* species have been extensively studied in the western countries. On the contrary the tropical countries, like India (especially the north western Himalayan region), are less explored. In the present study, the north-western Himalayan terrain of India was explored to document the diversity of *Suillus* species associated with conifer trees. A total of 40 specimens belonging to seven different *Suillus* species were examined in the present study. Out of seven species identified, three taxa namely, *S. triacicularis* sp. nov., *S. indicus* sp. nov., and *S. himalayensis* sp. nov. were found new to the science while *S. flavidus* and *S. placidus* were new records from India. *Suillus sibiricus* and *Suillus granulatus* were re-reported and described molecularly for the first time from India. Ecological data regarding abundance, distribution and association was also collected for these specimens. The observations suggested that *S. sibiricus* is the most abundant, frequently encountered and widely distributed *Suillus* species followed by *S. granulatus* and *S. triacicularis*. *Suillus sibiricus*, *S. granulatus* and *S. triacicularis* were found distributed throughout the northwestern Himalayan region of India ranging from Jammu and Kashmir to Uttarakhand, whereas only two specimens were recorded each for *S. himalayensis*, *S. indicus, S. flavidus* and *S. placidus*. *Suillus himalayensis* and *S. indicus* were observed only from the Barot Valley of Mandi district and Narkanda range of Shimla district of Himachal Pradesh, respectively, although probability of their occurrence from other localities can’t be denied. *Suillus flavidus* was found only in Himachal Pradesh as compared to *S. placidus*, which was collected both from Himachal Pradesh and Uttarakhand. The field experience suggested that *Suillus* species are mainly associated with the members of family *Pinaceae*. Most of them were found associated with *Pinus wallichiana*, but some also with *Pinus roxburghii*, *Cedrus deodara* and *Picea smithiana* trees. *Suillus triacicularis* was found fruiting exclusively in association with *P. roxburghii* trees. Although the species within the genus *Suillus* can be distinguished by their distinctive morphological characters, but the delimitation of *Suillus* species solely on the basis of morphological characters remains a challenge. Therefore, in the present study a phylogenetic analysis using ITS sequences was also performed. The consensus tree revealed that ITS sequences of *S. triacicularis* sp. nov., *S. indicus* sp. nov., and *S. himalayensis* sp. nov. clearly formed independent clades within the group, whereas those of *Suillus granulatus*, *Suillus sibiricus*, *S. flavidus* and *S. placidus* were clustered to respective clades along with their homologous sequences available in the database.

A total of eight cultures were isolated from the *Suillus* basidiocarps collected during the present study. ITS-RFLP analysis showed inter-specific genetic variations among these isolates and grouped them into five different ITS-RFLP taxa. One isolate was obtained each for *S. triacicularis* sp. nov., *S. indicus* sp. nov., *S. himalayensis* sp. nov. and *S. granulatus*, and four isolates were obtained for *S. sibiricus*. Some physiological variables of different *Suillus* isolates obtained in the present study were evaluated in order to select suitable isolates for mass inoculum production. All of the isolates were examined for their radial growth, biomass yield, extracellular enzyme activities, and *in vitro* mycorrhizal abilities with *P. wallichiana* seedlings. Significant inter-specific differences were found in radial growth and mycelia dry weight of *Suillus* isolates when grown under *in vitro* conditions. 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. 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. Variations in the root colonization were also observed within the isolates of *Suillus* species. In addition, different extracellular enzyme activities of ECM fungi primarily involved in nitrogen and phosphorus uptake were determined for all the *Suillus* isolates. It was observed that all the *Suillus* isolates showed acid phosphatase, phytase, protease and chitinase activities when grown *in vitro*. Significant differences were detected within the extracellular enzyme activities of different *Suillus* isolates. All these results revealed high physiological diversity, not only among different *Suillus* species (inter-specific) but also within the isolates of same species (intra-specific) i.e. *S. sibiricus*.

To select the efficient *Suillus* isolates for mass inoculum production and field inoculations, *in vitro* growth and nutrients content of ectomycorrhizal blue pine (*P. wallichiana*) seedlings were studied. Outcomes of the present investigation suggested that all the *Suillus* isolates tended to increase seedlings growth (seedlings height, root length, fresh weight and dry weight) and nutrient uptake (N, P, K, Mg and Ca) as compared to the un-inoculated control seedlings. In the present study, the maximum enhancement was recorded when inoculated with isolate *S. sibiricus* SNW06 followed by *S. indicus* SNW02 and therefore these two isolates were selected for further studies, such as mass inoculum production and nursery trial.

In the present research work, different medium composition and culture conditions were optimized with an aim to enhance the growth of selected *Suillus* isolates (*S. indicus* SNW02 and *S. sibiricus* SNW06) for mass inoculum production. Optimization of all these parameters led to development of two optimized media i.e. optimized medium 1 (OM1) for *S. indicus* SNW02 and optimized medium 2 (OM2) for *S. sibiricus* SNW06. Cultivation of both the selected *Suillus* isolates in respective optimized media resulted in a significant increase in the radial growth and biomass yield. For *S. indicus* SNW02, 1.58 fold increase was obtained in radial growth than the initial growth in 2% ME medium. In case of *S. sibiricus* SNW06, 1.54 fold increase was achieved in radial growth as compared to the initial growth in 2% ME medium. Moreover, the biomass yield increased 1.46 fold in *S. indicus* SNW02 and 1.39 fold in *S. sibiricus* SNW06 as compared to the initial yield. Thus, the optimization of culture conditions have remarkably increased the radial growth as well as the biomass yield of both the selected *Suillus* isolates, which is crucial for mass inoculum production in forestry practices.

To study the effects of selected *Suillus* isolates (*S. indicus* SNW02 and *S. sibiricus* SNW06) and biochar amendment on plant growth and rhizosphere properties, a nursery was established in February 2013 at Deeb (1830 m), Kumarsain (31.32°N 77.45°E), Shimla, Himachal Pradesh, India. Ectomycorrhizal root colonization, plant growth and nutrients content of all the sampled *P. wallichiana* seedlings were determined. The results revealed that biochar treatment showed slight increase in mycorrhizal root colonization in comparison to the non-biochar treatments. BC + *S. sibiricus* SNW06 treatment showed the highest root colonization as compared to all other treatments. Single mycorrhizal treatments, biochar treatment as well as biochar plus mycorrhizal treatments tended to increase the plant growth and nutrients content as compared to un-inoculated control and the results varied significantly across all the treatments. Among all the treatments, BC + *S. sibiricus* SNW06 treatment showed significantly higher plant growth and nutrients content followed by BC + *S. indicus* SNW02 treatment.

Further, the physico-chemical properties and enzyme activities of the sampled soils were also determined. It was found that single mycorrhizal, biochar and biochar plus mycorrhizal treatments altered the physico-chemical properties and enzyme activities of the soil. Only BC amendment was found to slightly increase the soil pH, whereas mycorrhizal inoculations with BC or without BC decreased the soil pH. The decrease in the soil pH was significantly higher in single mycorrhizal treatments as compared to combined treatments with biochar. In dual treatments of biochar and mycorrhizal fungi the decrease in soil pH was comparatively low. Both, mycorrhizal inoculation and biochar amendment raised the soil cation exchange capacity (CEC) as compared to the un-inoculated control. Increase in soil CEC was significantly higher in treatments with biochar addition (BC, BC + *S. indicus* SNW02 and BC + *S. sibiricus* SNW06) as compared to the single mycorrhizal treatments. Both mycorrhizal inoculants as well as biochar amendment tended to increase the total organic carbon (TOC) of soils. However, increase in TOC was significantly higher when treated with both biochar and mycorrhizal inoculants. Among the dual treatments, increase in TOC was significantly higher when treated with BC + *S. sibiricus* SNW06 as compared to BC + *S. indicus* SNW02. The content of total N, total P, and available P was also enhanced significantly in the treated soil as compared to the control. Dual treatments of biochar plus mycorrhizas were more effective than single mycorrhizal treatments in improving nutrients content of the treated soils. It was also observed that Total N, total P and available P contents of soil were highest when treated with BC + *S. sibiricus* SNW06 as compared to other treatments. Determination of enzyme activities of the soils revealed that both biochar amendment and mycorrhizal inoculations increased all the enzyme activities but the increase was higher in dual treatments (biochar amendment plus mycorrhizal fungi) than in single mycorrhizal treatments. Further, it was also observed that BC + *S. sibiricus* SNW06 treatment showed significantly higher increase in soil enzyme activities as compared to all other treatments.

Thus, the nursery trial suggested that biochar application to the soil, especially along with mycorrhizal inoculations, was advantageous for improvement of growth and nutrients content of blue pine seedlings as well as soil physico-chemical properties and enzyme activities. As a result of these findings, use of biochar as soil amendment in combination with mycorrhizal inoculants is suggested fruitful and desirable for forestation programmes.

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Appendix I

2% CTAB Buffer (pH 8.0)

CTAB	2% (w/v)
Tris-HCl	0.1 M
EDTA	20 mM
NaCl	1.4 M
2-mercaptoethanol	0.5% (w/v)

6× Gel Loading Dye

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Glycerol	30.0% (v/v)

Lacto phenol Cotton Blue

Ingredients	**Quantity (per litre)**
Phenol	200 g
Cotton Blue	0.5 g
Glycerol	400 ml
Lactic acid	200 ml
Deionized water	200 ml

LB-Ampicillin X-Gal/IPTG Agar Plates (pH 7.0±0.2)

Ingredients	**Quantity (g/l)**
NaCl	5.0
Yeast extract	5.0
Casein enzymic hydrolysate	10.0
Agar	15.0

Autoclaved at 15 lbs pressure (121 °C) for 15 min and then cooled to 55°C. Before pouring, 1 ml of filter sterilized ampicillin stock solution (50 mg/ml) was added to a final concentration of 50 µg/ml. To the solidified plates, 40 µl of X-Gal stock solution (20 mg/ml) and 40 µl of IPTG (100 mM) were added and then spreaded evenly over the medium with a sterile glass spreader.

Ligation reaction of amplicon in pTZ57R/T

Plasmid pTZ57R/T (55 ng/µl)	3.0 µl
Amplicon (50 ng/µl)	2.4 µl
Buffer (5X)	6.0 µl
T4 Ligase	1.0 µl
mΩH_2O	17.6 µl

Luria-Bertani (LB) Broth Medium (pH 7.0±0.2)

Ingredients	**Quantity (g/l)**
NaCl	5.0
Yeast extract	5.0
Casein enzymic hydrolysate	10.0

Autoclaved at 15 lbs pressure (121 °C) for 15 min and then cooled to 55°C before pouring.

Malt Extract (ME) Agar Medium

Ingredients	**Quantity (g/l)**
Malt Extract	20.0
Agar	15.0

pH was set to 5.5 using 0.1 M NaOH before adding agar and then autoclaved at 15 lbs pressure (121 °C) for 15 min. The medium was cooled to 55°C before pouring.

Melzer's reagent

Ingredients	Quantity (g/100ml)
Potassium iodide	5.0 g
Chloral hydrate	100.0 g
Iodine	1.5 g
Distilled water	upto 100 ml

Modified Melin-Norkrans (MMN) Medium

Ingredients	Quantity (per litre)
D-Glucose	10.0 g
Malt Extract	3.0 g
KH_2PO_4	0.5 g
$(NH_4)_2HPO_4$	0.25 g
$CaCl_2.2H_2O$	0.05 g
NaCl	0.025 g
$MgSO_4.7H_2O$	0.15 g
$FeCl_3$ (1%)	1.2 ml
Thiamine HCl	0.1 mg

pH was set to 5.5 using 0.1 N HCl and then autoclaved at 15 lbs pressure (121 °C) for 15 min. The medium was cooled to 55°C before pouring.

PEG Solution

Polyethylene glycol-6,000	20.0 % (w/v)
NaCl	2.5 M

Plasmid Extraction Solution I (pH 8.0)

Tris-HCl	25 mM
Glucose	50 mM
Na_2EDTA	10 mM

Plasmid Extraction Solution II

NaOH	2 M
SDS	10% (w/v)

Plasmid Extraction Solution III (pH 4.8)

5.0 M Potassium acetate

Potato Dextrose Agar (PDA) Medium

Ingredients	**Quantity (g/L)**
Infusion from potatoes	200
Dextrose	20.0
Agar	15.0

pH was set to 5.5 using 0.1 M HCl before adding agar and then autoclaved at 15 lbs pressure (121 °C) for 15 min. The medium was cooled to 55°C before pouring.

TBE Buffer (5×, pH 8.0)

Tris-HCl	0.45 M
Boric acid	0.45 M
Na_2EDTA	0.01 M

TE Buffer (1×, pH 8.0)

Tris-HCl	10.0 mM
Na_2EDTA	01.0 mM

Wash Buffer (pH 8.0)

Tris-HCl	0.1 M
2-mercaptoethanol	2% (w/v)
Polyvinylpyrrolidone	1% (w/v)
Ascorbic acid	0.05 M

Appendix II

ITS sequences of collection no. SHP11 (GenBank accession number: KF977188)

>*Suillus triacicularis* voucher PUN5534: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAAATTATAATTCGGCGAGGGAAAGGC
GGAGAGTTGTAGCTGGCCGCCTAGGCATGTGCACGCTCTCTTCTGAACTTTTGTCGTTA
TGGGCGTGGGGGGGCGACCCTCCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTC
GAATGTTATCATCATCATCGAGTCGCGACTTCTAGGAGACGCGATTCTTTGAGACAAAA
GTTATTACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGA
ATCGCGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCAC
CTTGCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACC
CCCCTCGATTTGCTTCGAGAGGGTGCTTGGATGGTGGAGGCTGCCGGAGACCTGGATTC
GTTCAGGACTTGGGCTCCTCTGAAATGAATCGGCTTGCGGTCGACTTTCGACTTTGCAT
GACAAGGCCTTTTGGCGTGATAATGATCGCCGTTCGCCGAAGTGCACGACCGAATCGTC
CCGCGCCTCTAATGCGTCGACGCCTTCTGGCGTCTTCCTTATTGACGTTTGACCTCAAA
TCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
```

ITS sequences of collection no. SHP27 (GenBank accession number: KF977189)

>*Suillus triacicularis* voucher PUN5538/isolate MTCC11954: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAAATTACAATTCGGCGAGGGAAAGGC
GGAGAGTTGTAGCTGGCCGCCTAGGCATGTGCACGCTCTCTTCTGAACTTTTGTCGTTA
TGGGCGTGGGGGGGGGCGACCCTCCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCT
TCGAATGTTATTATCATCATCGAGTCGCGACTTCTAGGAGACGCGATTCTTTGAGACAA
AAGTTATTACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGC
GAATCGCGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGC
ACCTTGCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAA
CCCCCCTCGATTTGCTTCGAGAGGGTGCTTGGATGGTGGAGGCTGCCGGAGACCTGGAT
TCGTTCAGGACTTGGGCTCCTCTGAAATGAATCGGCTTGCGGTCGACTTTCGACTTTGC
ATGACAAGGCCTTTTGGCGTGATAATGATCGCCGTTCGCCGAAGTGCACGACCGAATCG
TCCCGCGCCTCTAATGCGTCGACGCCTTCTGGCGTCTTCCTTATTGACGTTTGACCTCA
AATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
```

ITS sequences of collection no. SUK3 (GenBank accession number: KM882907)

>*Suillus triacicularis* voucher PUN5527: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAAATTACAATTCGGCGAGGGAAAGGC
GGAGAGTTGTAGCTGGCCGCCTAGGCATGTGCACGCTCTCTTCTGAACTTTTGTCGTTA
TGGGCGTGGGGGGGGGCGACCCTCCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCT
TCGAATGTTATTATCATCATCGAGTCGCGACTTCTAGGAGACGCGATTCTTTGAGACAA
AAGTTATTACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGC
GAATCGCGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGC
ACCTTGCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAA
CCCCCCTCGATTTGCTTCGAGAGGGTGCTTGGATGGTGGAGGCTGCCGGAGACCTGGAT
TCGTTCAGGACTTGGGCTCCTCTGAAATGAATCGGCTTGCGGTCGACTTTCGACTTTGC
ATGACAAGGCCTTTTGGCGTGATAATGATCGCCGTTCGCCGAAGTGCACGACCGAATCG
TCCCGCGCCTCTAATGCGTCGACGCCTTCTGGCGTCTTCCTTATTGACGTTTGACCTCA
AATCACGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
```

ITS sequences of collection no. SJK11 (GenBank accession number: KM882908)

>*Suillus triacicularis* voucher PUN5523: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAAATTATAATTCGGCGAGGGAAAGGC
GGAGAGTTGTAGCTGGCCGCCTAGGCATGTGCACGCTCTCTTCTGAACTTTTGTCGTTA
TGGGCGTGGGGGGGCGACCCTCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTCG
AATGTTATCATCATCATCGAGTCGCGACTTCTAGGAGACGCGATTCTTTGAGACAAAAG
TTATTACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAA
TCGCGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACC
TTGCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCC
CCCTCGATTTGCTTCGAGAGGGTGCTTGGATGGTGGAGGCTGCCGGAGACCTGGATTCG
TTCAGGACTTGGGCTCCTCTGAAATGAATCGGCTTGCGGTCGACTTTCGACTTTGCATG
ACAAGGCCTTTTGGCGTGATAATGATCGCCGTTCGCCGAAGTGCACGACCGAATCGTCC
CGCGCCTCTAATGCGTCGATGCCTTCTGGCGTCTTCCTTATTGACGTTTGACCTCAAAT
CAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG
```

ITS sequences of collection no. SHP07 (GenBank accession number: KJ675500)

>*Suillus indicus* voucher PUN6578/isolate MTCC11955: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
TTCGTAGGTGAACCTGCGGAAGGATCATTAACGAAATTATAATCCGGCGAGGGGAAGGC
GGCAGAGCTGTGGCTGGCCTCTTTACCGAGGCATGTGCACGCTCTCTTCTGGACTTTCG
CCGTATGGGCGTGTGGGGGCCTGCCCCCCCCGCGTCTTCATATACCTCTTCGTGTAGAA
AGTCTTTGAATGTTTTTACCATCATCGAGTCGCGACTTCTAGGAGACGCGATTCTTTGA
GACAAAAGTTATTACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAAC
GCAGCGAATCGCGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTG
AACGCACCTTGCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGTGTCAGTGAATT
CTCAACCCCTCTCGATTTGCTTCGAGCGGGTGCTTGGATCGTGGGGGCTGCCGGAGACC
TGGATTTATTCAGGACTCGGGCTCCCCTGAAATGCATCGGCTTGCGGTCGACTTTCGAC
TTTGCGCGACAAGGCCTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATGAACGA
ATGGGTCCCGTGCCTCCAATGCGTCGACGCCTTCTGGCGTCTTCCTCGTTGACTTTTGA
CCTCAAATCAGGTTGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG
```

ITS sequences of collection no. SHP03 (GenBank accession number: KJ675502)

>*Suillus indicus* voucher PUN6576: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
TTCGTAGGTGAACCTGCGGAAGGATCATTAACGAAATTATAATCCGGCGAGGGGAAGGC
GGCAGAGCTGTGGCTGGCCTCTTTACCGAGGCATGTGCACGCTCTCTTCTGGACTTTCG
CCGTATGGGCGTGTGGGGGCCTGCCCCCCCCGCGTCTTCATATACCTCTTCGTGTAGAA
AGTCTTTGAATGTTTTTACCATCATCGAGTCGCGACTTCTAGGAGACGCGATTCTTTGA
GACAAAAGTTATTACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAAC
GCAGCGAATCGCGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTG
AACGCACCTTGCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGTGTCAGTGAATT
CTCAACCCCTCTCGATTTGCTTCGAGCGGGTGCTTGGATCGTGGGGGCTGCCGGAGACC
TGGATTTATTCAGGACTCGGGCTCCCCTGAAATGCATCGGCTTGCGGTCGACTTTCGAC
TTTGCGCGACAAGGCCTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATGAACGA
AAGGTCCCGTGCCTCCAATGCGTCGACGCCTTCCGGCGTCTTCCTCGTTGACTTTTGAC
CTCAAATCAGGTTGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
```

ITS sequences of collection no. SHP15 (GenBank accession number: KF699850)

>*Suillus himalayensis* voucher PUN5535: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTTATAATCCGGCGAGACGAAGGA
CGAGAACTGTCGCTGGCCTTTCATCGAGGCATGTGCACGCTCTCTTCTGGACTTTCGCC
GTATGGGCGCGGGGCCACCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTTGAAT
GTTTTTACAATCATCGAGTCGTGACTTCTAGGAGACGCGATTCTTTGAGACAAAAGTTA
TCACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATCG
CGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCTC
TCGATTTGCTTCGAGTGGGGCTTGGATAGTGGGGGCTGCCGGAGACCTGGAATTCATTC
TGGGACTCGGGCTCCTCTGAAATGCATCGGCTTGCGGTCGACTTTCGACTTTGCGCGAC
AAAGCTTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATGAATGAATGGTCCCGT
GCCTCTAATCAGTCGACGCCTTCTGGGCGTCTTCCTTATAGACGTTTGACCTCAAATCA
GGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
```

ITS sequences of collection no. SHP26 (GenBank accession number: KJ472765)

>*Suillus himalayensis* voucher PUN5537/isolate MTCC11956: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTTATAATCCGGCGAGACGAAGGA
CGAGGACTGTCGCTGGCCTTTCATCGAGGCATGTGCACGCTCTCTTCTGGACTTTCGCC
GTATGGGCGCGGGGCCACCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTTGAAT
GTTTTTACAATCATCGAGTCGTGACTTCTAGGAGACGCGATTCTTTGAGACAAAAGTTA
TCACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATCG
CGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCTC
TCGATTTGCTTCGAGTGGGGCTTGGATAGTGGGGGCTGCCGGAGACCTGGAATTCATTC
TGGGACTCGGGCTCCTCTGAAATGCATCGGCTTGCGGTCGACTTTCGACTTTGCGCGAC
AAAGCTTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATGAATGAATGGTCCCGT
GCCTCTAATCAGTCGACGCCTTCTGGGCGTCTTCCTTATAGACGTTTGACCTCAAATCA
CGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
```

ITS sequences of collection no. SHP04 (GenBank accession number: KM882909)

>*Suillus granulatus* voucher PUN5533: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAAAAACATAATCTGGCGAGGGAAAGG
CGGAGAGTTGTAGCTGGCCTAGGCGCAACGTGCACGCTCTCTTCCGGACTTTCGTCGTA
TGGGCGTGGGGGGGCGACCCCTCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTAG
AATGTTATCATCATCATGGAGTCGCGACTTCCAGGAGACGCGATTCTTTGAGACAAAAG
TTATTACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAA
TCGCGATATGTAATGTGAATTGCAGATCTACAGTGTATCATCGAATCTTTGAACGCACC
TTGCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCC
CTCTCGATCTTTTTCGAGCGGGGCGCTTGGATGGTGGGGGCTGCCGGAGAACCTGGATT
CGTTCAGGACTCGGGCTCCTCTGAAATGAATCGGCTTGCGGTCGACTTTTCGACTTTGC
ACGACAAGGCCTTTGGCGTGATAATGATCGCCGTTCGCCGAAGTGCACGACCGAATCGT
CCCGTGCCTCTAATGCGTCGACGCCTTTTGGGGCGTCTTCCTTATCGACGTTTGACCTC
AAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

ITS sequences of collection no. SUK07 (GenBank accession number: KM882910)

>*Suillus granulatus* voucher PUN5530: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TCCGTAGGTGAACCTGCGGAAGGATCATTAATGAAAAACATAATCTGGCGAGGGAAAGG
CGGAGAGTTGTAGCTGGCCTAGGCAACGTGCACGCTCTCTTCCGGACTTTCGTCGTATG
GGCGTGGGGGGGCGACCCCTCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTAGAA
TGTTATCATCATCATGGAGTCGCGACTTCCAGGAGACGCGATTCTTTGAGACAAAAGTT
ATTACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATC
GCGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTT
GCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCT
CTCGATCTTTTTCGAGCGGGCGCTTGGATGGTGGGGGCTGCCGGAGAACCTGGATTCGT
TCAGGACTCGGGCTCCTCTGAAATGAATCGGCTTGCGGTCGACTTTTCGACTTTGCACG
ACAAGGCCTTTGGCGTGATAATGATCGCCGTTCGCCGAAGTGCACGACCGAATCGTCCC
GCGCCTCTAATGCGTCGACGCCTTTTGGGGCGTCTTCCTTATCGACGTTTGACCTCAAA
TCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

ITS sequences of collection no. SJK13 (GenBank accession number: KM882912)

>*Suillus granulatus* voucher PUN5525/isolate MTCC11957: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAAAAACATAATCTGGCGAGGGAAAGG
CGGAGAGTTGTAGCTGGCCTAGGCAACGTGCACGCTCTCTTCCGGACTTTCGTCGTATG
GGCGTGGGGGGGCGACCCCTCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTAGAA
TGTTATCATCATCATGGAGTCGCGACTTCCAGGAGACACGATTCTTTGAGACAAAAGTT
ATTACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATC
GCGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTT
GCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCT
CTCGATCTTTTTCGAGCGGGCGCTTGGATGGTGGGGGCTGCCGGAGAACCTGGATTTGT
TCAGGACTCGGGCTCCTCTGAAATGAATCGGCTTGCGGTCGACTTTTCGACTTTGCACG
ACAAGGCCTTTGGCGTGATAATGATCGCCGTTCGCCGAAGTGCACGACCGAATCGTCCC
GCGCCTCTAATGCGTCGACGCCTTTTGGGGCGTCTTCCTTATCGACGTTTGACCTCAAA
TCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

ITS sequences of collection no. SHP34 (GenBank accession number: KM882911)

>*Suillus granulatus* voucher PUN6585: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAAAAACATAATCTGGCGAGGGAAAGG
CGGAGAGTTGTAGCTGGCCTAGGCAACGTGCACGCTCTCTTCCGGACTTTCGTCGTATG
GGCGTGGGGGGGCGACCCCTCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTAGAA
TGTTATCATCATCATGGAGTCGCGACTTCCAGGAGACGCGATTCTTTGAGACAAAAGTT
ATTACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATC
GCGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTT
GCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCT
CTCGATCTTTTTCGAGCGGGCGCTTGGATGGTGGGGGCTGCCGGAGAACCTGGATTCGT
TCAGGACTCGGGCTCCTCTGAAATGAATCGGCTTGCGGTCGACTTTTCGACTTTGCACG
ACAAGGCCTTTGGCGTGATAATGATCGCCGTTCGCCGAAGTGCACGACCGAATCGTCCC
GCGCCTCTAATGCGTCGACGCCTTTTGGGGCGTCTTCCTTATCGACGTTTGACCTCAAA
TCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

ITS sequences of collection no. SJK01 (GenBank accession number: KM882913)

>*Suillus sibiricus* voucher PUN5520/isolate MTCC11958: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTTATAATCCGGCGAGACGAAGGA
CGAGGACTGTCGCTGGCCTTTCATCGAGGCATGTGCACGCTCTCTTCTGGACTTTCGCC
GTATGGGCGCGGGGCGACCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTTGAAT
GTTTTTACAATCATCGAGTCGTGACTTCTAGGAGACGCGATTCTTTGAGACAAAAGTTA
TCACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATCG
CGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCTC
TCGATTTGCTTCGAGTGGGGCTTGGATAGTGGGGGCTGCCGGAGACCTGGAATTCATTC
TGGGACTCGGGCTCCTCTGAAATGCATCGGCTTGCGGTCGACTTTCGACTTTGCGCGAC
AAAGCTTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATGAATGAATGGTCCCGT
GCCTCTAATCAGTCGACGCCTTCTGGGCGTCTTCCTTATAGACGTTTGACCTCAAATCA
GGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
```

ITS sequences of collection no. SHP05 (GenBank accession number: KM882914)

>*Suillus sibiricus* voucher PUN6577/isolate MTCC11959: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTTATAATCCGGCGAGACGAAGGA
CGAGGACTGTCGCTGGCCTTTCATCGAGGCATGTGCACGCTCTCTTCTGGACTTTCGCC
GTATGGGCGCGGGGCGACCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTTGAAT
GTTTTTACAATCATCGAGTCGTGACTTCTAGGAGACGCGATTCTTTGAGACAAAAGTTA
TCACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATCG
CGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCTC
TCGATTTGCTTCGAGTGGGGCTTGGATAGTGGGGGCTGCCGGAGACCTGGAATTCATTC
TGGGACTCGGGCTCCTCTGAAATGCATCGGCTTGCGGTCGACTTTCGACTTTGCGCGAC
AAAGCTTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATGAATGAATGGTCCCGT
GCCTCTAATCAGTCGACGCCTTCTGGGCGTCTTCCTTATAGACGTTTGACCTCAAATCA
GGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
```

ITS sequences of collection no. SHP12 (GenBank accession number: KM882919)

>*Suillus sibiricus* voucher PUN6579/isolate MTCC11960: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTTATAATCCGGCGAGACGAAGGA
CGAGGACTGTCGCTGGCCTTTCATCGAGGCATGTGCACGCTCTCTTCTGGACTTTTCGC
CGTATGGGCGCGGGGCGACCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTATGAA
TGTTTTTACAATCATCGAGTCGTGACTTCTAGGAGACGCGATTCTTTGAGACAAAAGTT
ATCACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATC
GCGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTT
GCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCT
CTCGATTTGCTTCGAGTGGGGCTTGGATAGTGGGGGCTGCCGGAGACCCAGAATTCATT
CTGGGACTTGGGCTCCTCTGAAATGCATCGGCTTGCGGTCGACTTTCGACTTTGCGCGA
CAAAGCTTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATGAATGAATGGTCCCG
TGCCTCTAATCAGTCGACGCCTTCTGGGCGTCTTCCTTATAGACGTTTGACCTCAAATC
AGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

ITS sequences of collection no. SHP02 (GenBank accession number: KM882915)

>*Suillus sibiricus* voucher PUN6575: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTTATAATCCGGCGAGACGAAGGA
CGAGGACTGTCGCTGGCCTTTCATCGAGGCATGTGCACGCTCTCTTCTGGACTTTCGCC
GTATGGGCGCGGGGCGACCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTTGAAT
GTTTTTACAATCATCGAGTCGTGACTTCTAGGAGACGCGATTCTTTGAGACAAAAGTTA
TCACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATCG
CGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCTC
TCGATTTGCTTCGAGTGGGGCTTGGATAGTGGGGGCTGCCGGAGACCTGGAATTCATTC
TGGGACTCGGGCTCCTCTGAAATGCATCGGCTTGCGGTCGACTTTCGACTTTGCGCGAC
AAAGCTTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATGAATGAATGGTCCCGT
GCCTCTAATCAGTCGACGCCTTCTGGGCGTCTTCCTTATAGACGTTTGACCTCAAATCA
GGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

ITS sequences of collection no. SUK20 (GenBank accession number: KM882916)

>*Suillus sibiricus* voucher PUN6569: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTTATAATCCGGCGAGACGAAGGA
CGAGGACTGTCGCTGGCCTTTCATCGAGGCATGTGCACGCTCTCTTCTGGACTTTCGCC
GTATGGGCGCGGGGCGACCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTTGAAT
GTTTTTACAATCATCGAGTCGTGACTTCTAGGAGACGCGATTCTTTGAGACAAAAGTTA
TCACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATCG
CGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCTC
TCGATTTGCTTCGAGTGGGGCTTGGATAGTGGGGGCTGCCGGAGACCTGGAATTCATTC
TGGGACTCGGGCTCCTCTGAAATGCATCGGCTTGCGGTCGACTTTCGACTTTGCGCGAC
AAAGCTTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATGAATGAATGGTCCCGT
GCCTCTAATCAGTCGACGCCTTCTGGGCGTCTTCCTTATAGACGTTTGACCTCAAATCA
GGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

ITS sequences of collection no. SUK12 (GenBank accession number: KM882917)

>*Suillus sibiricus* voucher PUN5532/isolate MTCC11961: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTTATAATCCGGCGAGACGAAGGA
CGAGGACTGTCGCTGGCCTTTCATCGAGGCATGTGCACGCTCTCTTCTGGACTTTCGCC
GTATGGGCGCGGGGCGACCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTTGAAT
GTTTTTACAATCATCGAGTCGTGACTTCTAGGAGACGCGATTCTTTGAGACAAAAGTTA
TCACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATCG
CGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCTC
TCGATTTGCTTCGAGTGGGGCTTGGATAGTGGGGGCTGCCGGAGACCTGGAATTCATTC
TGGGACTCGGGCTCCTCTGAAATGCATCGGCTTGCGGTCGACTTTCGACTTTGCGCGAC
AAAGCTTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATGAATGAATGGTCCCGT
GCCTCTAATCAGTCGACGCCTTCCGGGCGTCTTCCTTATAGACGTTTGACCTCAAATCA
GGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

ITS sequences of collection no. SJK03 (GenBank accession number: KM882918)

>*Suillus sibiricus* voucher PUN5522: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTTATAATCCGGCGAGACGAAGGA
CGAGGACTGTCGCTGGCCTTTCATCGAGGCATGTGCACGCTCTCTTCTGGACTTTCGCC
GTATGGGCGCGGGGCGACCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTTGAAT
GTTTTTACAATCATCGAGTCGTGACTTCTAGGAGACGCGATTCTTTGAGACAAAAGTTA
TCACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATCG
CGATATGTAATGTGAATTGCAGATCTACGGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCTC
TCGATTTGCTTCGAGTGGGGCTTGGATAGTGGGGGCTGCCGGAGACCTGGAATTCATTC
TGGGACTCGGGCTCCTCTGAAATGCATCGGCTTGCGGTCGACTTTCGACTTTGCGCGAC
AAAGCTTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATGAATGAATGGTCCCGT
GCCTCTAATCAGTCGACGCCTTCTGGGCGTCTTCCTTATAGACGTTTGACCTCAAATCA
GGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
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ITS sequences of collection no. SHP01 (GenBank accession number: KM882920)

>*Suillus flavidus* voucher PUN6574: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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TTCGTAGGTGAACCTGCGGAAGGATCATTAATGAATTTATAATCCGGCGAGACGAAGGA
CGAGGACTGTCGCTGGCCTTCAACCGAGGCATGTGCACGCTCTCTTCTGGACTTTCGCC
GTATGGGCGCGGGGCGACCCGCGTCTTCATATACCTCTTCGTGTAGAAAGTCTTCGAAT
GTTTTTACCATCATCGAGTCGCGACTTCTAGGAGACGCGATTCTTTGAGACAAAAGTTA
TCACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAATCG
CGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACCCCTC
TCGATTTGCTTCGAGTGGGGCTTGGATAGTGGGGGCTGCCGGAGACCTGAAATTCATTT
CGGGACTCGGGCTCCTCTGAAATGCATCGGCTTGCGGTCGACTTTCGACTTTGCGCGAC
AAAGCTTTCGGCGTGATAATGATCGCCGTTCGCTGAAGCGCATCAATGAACCGTCCCGT
GCCTCTAATCAGTCGACGCCTTCTGGGCGTCTTCCTTATAGACGTTTGACCTCAAATCA
GGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
```

ITS sequences of collection no. SHP32 (GenBank accession number: KM882921)

>*Suillus placidus* voucher PUN6584: 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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TTCGTAGGTGAACCTGCGGAAGGATCATTAACGAATTCATAATTCGGCGAGGGGAAAGC
GGAGGGTTGTAGCTGGCCTTTTTACCGAGGCACGTGCACGCTCTCTTCCGAACTCTCGT
CGTATGGGCGCGGGGCGACCCGCGTCTTTCATCCCACCTCTTCGTGTAGAAAGTCTTTG
AATGTTTTTACCATCATCGAGTCGCGACTTCTAGGAGACGCGATTCTTTGAGACAAAAG
TTTATTACAACTTTCAGCAATGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGA
ATCGCGATATGTAATGTGAATTGCAGATCTACAGTGAATCATCGAATCTTTGAACGCAC
CTTGCGCTCCTCGGTGTTCCGAGGAGCATGCCTGTTTGAGCGTCAGTAAATTCTCAACC
CCTCTCGATTTGCTTCGAGAGGGCGCTTGGATGGTGGGGGCTGCCGGAGACCTGGATTT
ATCCCTGGACTCGGGCTCTCCTGAAATGCATCGGCTTGCGGTCGACTTTCGACTTTGCG
CGACAAGGCCTTCGGCGTGATAATGATCGCCGTTCGCCGAAGCGCATGAATGAACGGTC
CCGCGCCTCTAATCCGTCGACGCTTTCGAGCGTCTTCCTCATTGACGTTTGACCTCAAA
TCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
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Suillus triacicularis sp. nov., a new species associated with *Pinus roxburghii* from northwestern Himalayas, India

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Abstract

A new *Suillus* species, *Suillus triacicularis sp. nov.*, is reported from northwestern Himalayan region of India. This is the first report of any *Suillus* species found in ectomycorrhizal association with *Pinus roxburghii* in this region. Morphologically, the species is very close to *Suillus granulatus* but can be distinguished mainly by the yellow to reddish or orange-yellow pileus color at maturity and the absence of watery green context next to the tubes. Phylogenetic analysis of internal transcribed spacer region revealed that this species is distinct from other closely related species of *Suillus*. Field photographs of fresh sporocarps, microscopic line drawings, and a phylogenetic tree are provided along with the taxonomic details.

Key words: Boletales, ectomycorrhizal, ITS, phylogeny, *Suillaceae*, taxonomy

Introduction

The genus *Suillus* was founded by S.F. Gray in Gray (1821: 646) and about 50 different species have been reported worldwide (Kirk *et al*. 2008). *Suillus* species are common root symbionts of the family *Pinaceae* (Kretzer *et al*. 1996, Wu *et al*. 2000) and have been reported by many authors (Timonen *et al*. 1997, Wallander 2000, González-Ochoa *et al*. 2003, Beatriz *et al*. 2006) to enhance pine growth, survival and nutrient uptake. The pioneer work on documenting *Suillus* diversity was done by Smith & Thiers (1964). Thereafter, several other investigations on diversity and evolutionary phylogenetics of *Suillus* species from different parts of the world have been reported (Corner 1972, Thiers 1979, Kretzer *et al*. 1996, Kretzer & Bruns 1997, Wu *et al*. 2000, Manian *et al*. 2001, Beatriz *et al*. 2006, Feng *et al*. 2008). Recently, a new *Suillus* species, *S. quiescens* T.D. Bruns & Vellinga in Bruns *et al*. (2010: 442), was described from California and Oregon. Very little is known about *Suillus* diversity from the Himalayan region of India. *Suillus sibiricus* (Singer) Singer (1945: 260) and *Suillus granulatus* (L.) Roussel in Roussel (1796: 34) have been reported from Himachal Pradesh and Jammu & Kashmir states of India, respectively (Sagar & Lakhanpal 2005, Dar *et al*. 2010). During our field surveys in northwestern Himalayas (2009–2013), both these *Suillus* species were found associated mainly with *Pinus wallichiana* A.B. Jacks. in Jackson (1938: 85) and seldom with *Cedrus deodara* (Roxb. ex D. Don) G. Don in Loudon (1830: 388). So far, there is no record for any *Suillus* species colonizing *Pinus roxburghii* Sarg. in Sargent (1897: 9).

Pinus roxburghii, commonly known as Chir pine, is a pine native to the Himalayas. It is found at low elevations ranging from 450–2300 m, extending longitudinally from 71°–93°E and latitudinally from 26°–36°N, and is distributed over an area of 8900 sq. km in India (Arya *et al*. 2000, Sharma & Lekha 2013). In general they develop as pure stands, but often form mixed stands with other species especially at their outer limits. Chir pine is an extensively used pine species for afforestation programmes due to its traditional, medicinal, and industrial utilities. Considering the commercial plantations and afforestation of Chir pine, the ectomycorrhizal community associated with the pine is also of utmost importance and needs to be documented and conserved. In the present study, we describe a new and rare *Suillus* species from Chir pine forests in northwestern Himalayas that was found fruiting exclusively in association with *P. roxburghii* trees. Morphological examination and phylogenetic analysis

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of the specimens confirmed that they belong to a species which is different from all known species of *Suillus* and therefore, we describe this species as new.

Materials and Methods

Study sites

The sporocarps were collected from five different localities of the northwestern Himalayas ranging from Jammu and Kashmir to Uttarakhand, namely Kud (latitude 33.08ºN and longitude 75.28ºE), Palampur (latitude 32.12°N and longitude 76.53°E), Karsog (latitude 31.38°N and longitude 77.20°E), Mussoorie (latitude 30.45°N and longitude 78.08°E), and Nainital (latitude 29.23°N and longitude 79.27°E), respectively. The specimens were collected under canopies of *P. roxburghii*, which is the most abundant pine species in northwestern Himalayas.

Morphological description

Specimens have been deposited in the Herbarium of the Botany Department (PUN), Punjabi University, Patiala, India under the voucher numbers PUN5523, PUN5527, PUN5531, PUN5534, and PUN5538. The specimens were described taxonomically following Corner (1972). Morphological details and chemical color reactions were noted in the field and the color codes used for describing sporocarp parts are in accordance with Kornerup & Wanscher (1978). Microscopic line drawings were made using a camera lucida. Basidiospore measurements were made using a calibrated ocular micrometer on an Olympus light microscope at 1000x magnifications and exclude the length of the apiculus and basidium length excludes the length of the sterigmata. Quotient value (Q=L/W) for spores was calculated considering the mean value of length and width of 20 basidiospores.

Molecular and phylogenetic analysis

Genomic DNA from dried specimens was isolated as described by Zhou *et al*. (1999) and quantified with a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). Nuclear internal transcribed spacer (ITS) region was amplified using primer pair ITS1 and ITS4 (White *et al*. 1990). The 50 µl reaction mixture consisted of 100 ng genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer and 2.5 units of *i*-taq^{TM} DNA polymerase (iNtRON Biotechnology, KOREA). Amplification reactions were performed in a PCR Thermal Cycler (Perkin Elmer, USA) with initial denaturation of 3 min at 95ºC, followed by 34 cycles of 1 min at 94ºC, 1 min at 50ºC, and 1.5 min at 72ºC and a final extension of 7 min at 72ºC. PCR products were purified with Qiaquick columns (Qiagen) and sub-cloned using InsTAclone, PCR cloning kit (Thermo Scientific), as per the manufacturer's instructions. Subsequently, the *Escherichia coli* DH5α cells were transformed and clones containing the ITS insert were selected randomly and sequenced. The sequences have been deposited in GenBank under the accession numbers KF977188 and KF977189.

The ITS sequences obtained in the present study were compared to those in the GenBank database using the nucleotide BLAST search algorithm. Based on the BLAST results and the outcomes of recent phylogenetic study on *Suillus* (Bruns *et al*. 2010), sequences belonging to the granulatus-like group were selected for phylogenetic analysis. The sequences were aligned using MAFFT ver. 7.0 (Katoh & Standley 2013) and edited with BioEdit 5.0.6 (Hall 1999). The aligned data matrix was deposited at Tree BASE, which is available under the submission ID number 15111. Phylogenetic analysis was performed using Bayesian Inference (BI). The BI was performed with MrBayes v.3.2.2 (Ronquist *et al*. 2011) with two parallel runs of four incrementally heated simultaneous Monte Carlo Markov Chains over 2 million generations. The sample frequency was set to 100, resulting in an overall sampling of 20,000 trees and the first 5,000 trees were discarded as "burn-in" (0.25). For the remaining trees, a majority rule consensus tree was computed to obtain estimates for Bayesian posterior probabilities (BPP).

Results

Molecular Phylogeny

BLASTn search for the sequences derived in the present study showed maximum similarity (95% for 99%

query coverage) with *S. collinitus* isolate CCMA-79 (Beatriz *et al.* 2006). The ITS matrix for Bayesian analysis consisted of 54 taxa representing homologous ITS sequences of 17 closely related granulatus-like *Suillus* species. The alignment resulted in a data matrix comprising 690 characters including gaps. Bayesian analysis of the ITS region yielded a consensus tree as shown in Fig. 1. The two ITS sequences of the present study (*S. triacicularis*) form a well-supported clade when compared with ITS sequences of other *Suillus* species. Bayesian consensus showed splits between *S. triacicularis* and a well-resolved clade containing *S. collinitus* (Fr.) Kuntze in Kuntze (1898: 536) and *S. granulatus*, the two species that it macroscopically resembles. Posterior probability percentage (99%) supports the species as a separate clade in the group. A few pairs of *Suillus* species (*S. pseudobrevipes* and *S. volcanalis*, *S. glandulosipes* and *S. neoalbidipes*, *S. brevipes* and *S. weaverae*) are not distinguishable by the ITS locus. On the contrary, the ITS sequences of *S. granulatus* derived from Europe (L54076, AY898617, DQ068968, EF493251, EF493252, AJ272409, AJ272408, & AJ272410), Asia (L54121), and North America (L54113) are polyphyletic and sub-divided into three different clades (SG1, SG2, & SG3, Fig. 1).

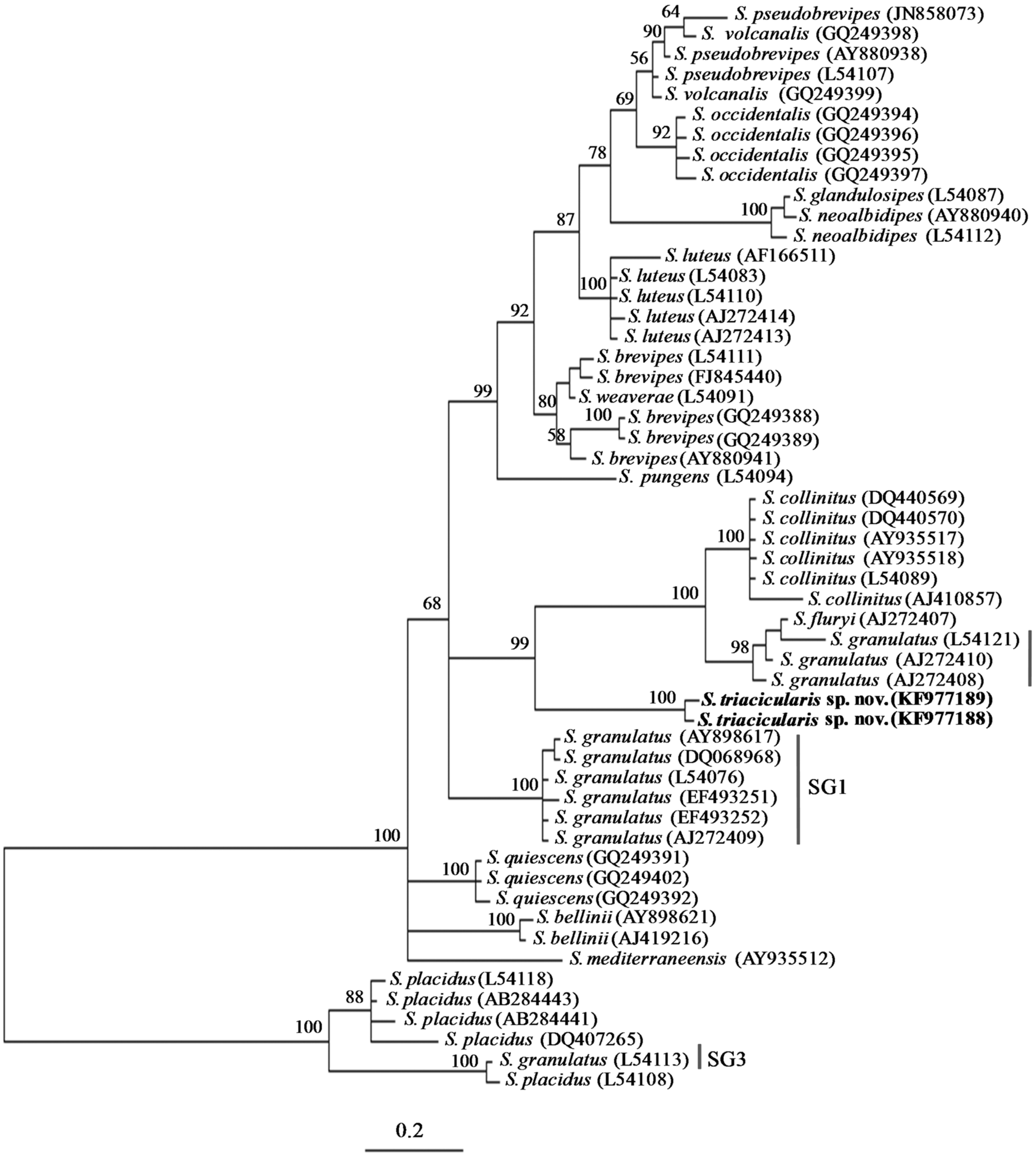

FIGURE 1. Bayesian analysis of ITS sequences for granulatus-like group of *Suillus*. Bold indicates *Suillus triacicularis sp. nov.* Bayesian posterior probability (BPP) percentages above 50% are shown at the nodes of the tree. SG1 (European), SG2 (European & Asian) and SG3 (North American) refer to different groups including *S. granulatus* isolates.

Taxonomy

Suillus triacicularis B. Verma & M.S. Reddy, *sp. nov.* (Figs. 2 & 3) MycoBank MB 807281

Diagnosis:—The species differs from *Suillus granulatus* by the absence of watery green context above the tubes during any stage of development. The absence of cloudy droplets over the tubes when young and the yellow to reddish or orange-yellow pileus color at maturity also distinguish it from *Suillus granulatus*.

Type:—INDIA. Himachal Pradesh: Karsog, 1404 m, 5th August 2011, *B. Verma* (PUN5534, Holotype!).

Basidiocarps 5.0–8.5 cm in size. Pileus 4.0–8.0 cm broad, convex when young, flattened with age; margin regular, strongly incurved when young, irregular and undulating with maturity; pileal veil absent at any stage of development; pileus surface dry to moist, slightly viscid during rainy season, glabrous, yellowish white (1A2) to pale yellow (1A3) when young turning yellow (2A6–2A7) then to reddish or orange yellow with age (4B7–4B8), cuticle fully peeling, umbo absent; scales absent; pileus context concolorous with pileus (1A2–1A3), unchanging, slowly turning light yellow then to yellow (2A4–2A6) on exposure, 5–15 mm thick; odor not distinctive and taste mild. Tubes 1–5 mm deep, decurrent, radial, crowded, fully peeling, yellowish grey to greyish yellow (2B2–2B6), unchanging, spotted light brown to brown with age; pore mouths angular, 1–2 per mm. Stipe central, 3.0–6.5 cm long, 8–12 mm thick, tubular and equal in diameter, concolorous with pileus (1A2–1A3), unchanging with age or when bruised, covered with orange red to reddish brown (8A8–8D8) glandular dots and smears throughout but fewer at base; annulus absent; white mycelium at the base. Stipe context pale yellow (1A3), unchanging, solid, becoming hollow with age.

FIGURE 2. *Suillus triacicularis* (PUN5534, holotype!). a. Young sporocarps with yellowish white to pale yellow pileus. b. Mature sporocarps showing yellow to reddish or orange-yellow pileus. c. Light brown to brown spotted tubes. d. Orange-red to reddish brown glandular dots or smears on stipe. Scale bars: a–d=2 cm.

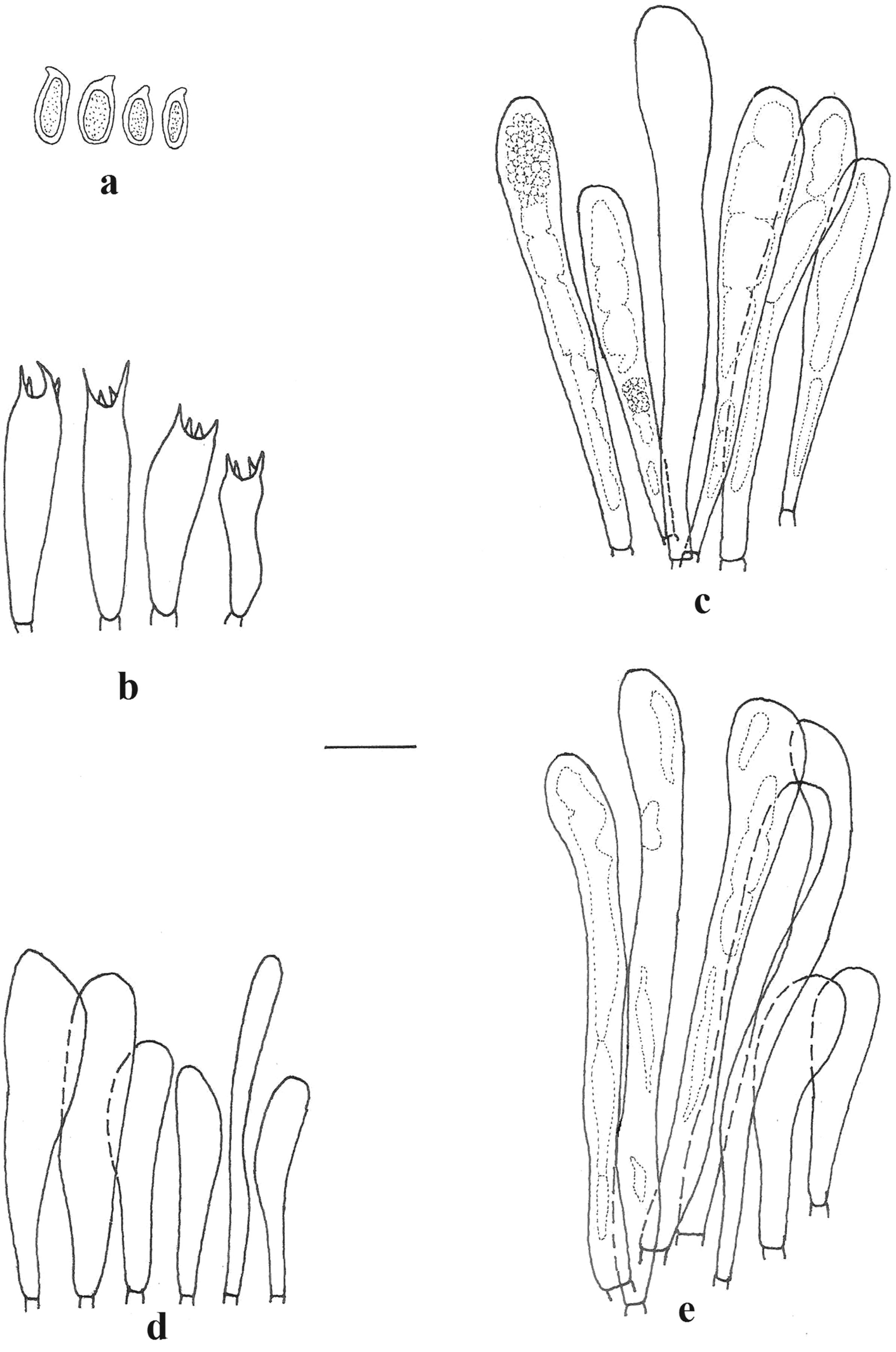

FIGURE 3. *Suillus triacicularis*, microscopic line drawings. a. Basidiospores. b. Basidia. c. Pleurocystidia. d. Cheilocystidia. e. Caulocystidia. Scale bar: a–e=10 µm.

Spore deposit light brown (6D8), spores 6.2–8.5 × 2.3–3.8 µm (Q=2.0), elongated or oblong in shape, nearly hyaline in KOH, pale yellow in Melzer's reagent, smooth, with granular content inside. Basidia 16.0–24.0 × 4.4–6.2 µm, clavate to cylindrical, granular, 4 spored, hyaline in KOH and yellowish in Melzer's reagent; sterigmata 1.5–3.9 µm high. Pleurocystidia numerous to abundant, 40.0–62.0 × 6.0–8.0 µm, cylindrical to subclavate, in fascicles or scattered, hyaline with uniform brown coagulated contents in KOH, yellowish with brownish contents in Melzer' reagent. Cheilocystidia 24.5–38.5 × 3.0–9.3 µm, mostly in fascicles, hyaline in KOH and yellowish in Melzer's reagent, brown content absent. Caulocystidia 26.0–70.0 × 6.0–9.3 µm, almost similar to pleurocystidia. Trama gelatinous and divergent. Clamp connections absent.

Chemical color reactions:—Pileal flesh: 2.5% KOH—reddish, 10% $FeSO_4$—grayish green, 14% ammonia—reddish then to reddish brown, conc. HNO_3—no color reaction. Pileus cuticle: 2.5% KOH—light brown then to blue black, 10% $FeSO_4$—olive gray, 14% ammonia—brown then to black brown, conc. HNO_3—no color reaction. Reddish color reaction of pileal flesh with KOH and ammonia identify it as a *Suillus* species and distinguish it from other *Boletaceae* (Baroni 1978).

Habitat and Distribution:—*Suillus triacicularis* is found fruiting solitary, scattered or gregarious under *P. roxburghii* trees during the monsoon season. Its distribution ranges with the natural distribution of *P. roxburghii* in the northwestern Himalayas. The occurrence of *S. triacicularis* is rare and the species is seldom found in pure stands of *P. roxburghii* in this geographical region. No records were found from other *Pinaceae* forests during our 5 years of excursions to these forests from 2009 to 2013.

Etymology:—The specific epithet "*triacicularis*" is derived from Latin and refers to the fact that the new species occurs in association with a 'three-needle' pine.

Other collections examined:—INDIA. Himachal Pradesh: Palampur, 1472 m, 29th July 2012, *B. Verma* (PUN5538!). Uttarakhand: Mussoorie, 1825 m, 30th July 2011, *B. Verma* (PUN5527!); Nainital, 2084 m, 20th August 2010, *B. Verma* (PUN5531!). Jammu and Kashmir: Udhampur; Kud, 1855 m, 18th July 2010, *B. Verma* (PUN5523!).

Discussion

Morphologically, *S. triacicularis* resembles *S. granulatus* and *S. collinitus*. The absence of watery green context above the tubes during any stage of development distinguishes it from *S. granulatus*. The species looks very similar to pallid variants of *S. granulatus* when young, but differs by absence of cloudy droplets over the tubes. Moreover, the yellow to reddish or orange-yellow pileus at maturity separates it from *S. granulatus*, which has a cinnamon brown pileus at maturity. *S. collinitus*, which is very similar to *S. granulatus*, can be distinguished by its pinkish mycelia at the stipe base as compared to pure white mycelia in *S. granulatus* and *S. triacicularis*.

As mentioned earlier by Bruns *et al.* (2010), a few pairs of *Suillus* species (*S. pseudobrevipes* and *S. volcanalis*, *S. glandulosipes* and *S. neoalbidipes*, *S. brevipes* and *S. weaverae*) are not distinguished by the ITS locus. This might be due to over-description (=synonymy) or to the lack of ITS divergence among sibling species. Thus, there are limitations of using ITS locus for species-level determinations in the genus *Suillus*. Further, the isolates of *S. granulatus* are separated into different groups, probably suggesting that a cryptic speciation process has taken place between these isolates. Heterogeneity among *S. granulatus* isolates on the basis of molecular data has been also observed by Kretzer *et al.* (1996), Manian *et al.* (2001), and Bruns *et al.* (2010) and it seems that isolates reported as *S. granulatus* from Europe, Asia, and North America might represent at least two different taxa. In the phylogenetic analysis of the ITS locus (Fig. 1), *S. triacicularis* occupies an independent position and is sister to the *S. collinitus* and *S. granulatus* (SG2) clades. These results show that *S. triacicularis* is clearly distinct from other closely related species of *Suillus*. Based on morphological characters as well as the molecular analysis of the ITS region, a new *Suillus* species, *S. triacicularis* is proposed.

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Nova Hedwigia Vol. 99 (2014) Issue 3–4, 541–550 **Article**

Suillus himalayensis (Basidiomycota, Agaricomycetes, Boletales), a new species associated with *Pinus wallichiana* from the northwestern Himalayas, India

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With 3 figures and 1 table

Abstract: *Suillus himalayensis* sp. nov. is found in ectomycorrhizal association with *Pinus wallichiana* throughout the Barot valley in the district Mandi of Himachal Pradesh, India. The species can be distinguished morphologically by the yellow white to pale yellow pileus surface with light greenish tinge and brownish squamules, and the flesh color changing to grayish green and tubes to olive brown on bruising. The sequence analysis of the internal transcribed spacer region and the combination of morphological characters studied identify this as a distinct species from other closely related species of *Suillus*.

Key words: Ectomycorrhizal, Himalayan, ITS, *Suillaceae*, Taxonomy.

Introduction

Suillus S.F.Gray is an ectomycorrhizal fungi belonging to the family *Suillaceae*. They are found in temperate, boreal, and Mediterranean forests mainly associated with the members of family *Pinaceae* and also with some deciduous species (Singer 1986, Kretzer et al. 1996, Wu et al. 2000). Many studies have shown that *Suillus* spp. improve the plant growth, survival, mineral nutrition, (Timonen et al. 1996, 1997; Wallander 2000; González et al. 2003; El Karkouri et al. 2006; Beatriz et al. 2006) and also provide tolerance against the heavy metal toxicity in pine seedlings (Colpaert et al. 2000, 2004; Adriaensen et al. 2003, 2005; Krznaric et al. 2009). Owing to their important role in forest ecology and rehabilitation, considerable research has been done on their diversity and evolutionary phylogenetics from many parts of the world (Smith and Thiers 1964, Corner 1972, Thiers 1979, Kretzer et al. 1996, Kretzer and Bruns 1997, Wu et al. 2000, Manian et al. 2001, Beatriz et al. 2006, Feng et al. 2008).

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About 50 different species of *Suillus* has been documented throughout the world so far (Kirk et al. 2008). Recently, a new species *Suillus quiescens* T.D.Bruns & E.C.Vellinga was reported from California and Oregon (Bruns et al. 2010).

Several investigations on phylogenetics and bio-geographical analysis of the genus *Suillus* have demonstrated the advantage of using nuclear ribosomal RNA (nrRNA) region to diagnose and figure out the phylogenetic relationships among the species in this genus (Kretzer et al. 1996, Wu et al. 2000, Manian et al. 2001, Feng et al. 2008). In the present study, the internal transcribed spacer (ITS) region of the collected specimens was analyzed because the ITS region has been sequenced for large number of species in the genus *Suillus*. We describe *Suillus himalayensis,* a new species to science that we have collected from the Barot valley during the early monsoon season fruiting in association with *Pinus wallichiana* along with the population of *Suillus sibiricus* (Singer) Singer. Morphological and molecular comparison of the specimens with morphologically similar and earlier diagnosed species of *Suillus* indicated this to be a new species of *Suillus* and has been designated as *Suillus himalayensis*.

Materials and methods

Collection sites and fungal isolation: Fresh basidiocarps were collected under *Pinus wallichiana* trees from Lachkandi and Tikkan sub localities of Barot (latitude 32°2'11"N and longitude 76°50'51"E), Mandi, Himachal Pradesh, India. The vegetation of the Barot valley is dominated by *Cedrus deodara* and *Quercus* spp. with some randomly distributed stands of mixed and pure *P. wallichiana* forests along the bank of the river Uhl. Pure culture was obtained using basidiocarp tissue and grown on Malt Extract (ME) agar (2% w/v) media supplemented with streptomycin (50µg/ml). The pure culture isolated is being maintained on routine basis in our laboratory.

Morphological description: The taxonomic details of the examined collections were studied in accordance to the methodology given by Corner (1972). The terminology used for describing the color tone of the basidiocarp parts and spore print is as described in Kornerup & Wanscher (1978). Microscopic line drawings were made with the aid of a camera lucida at 1000×. Basidiospore measurements exclude the length of the apiculus. Basidium length excludes the length of the sterigmata. Quotient (Q=L/W) was calculated considering the mean value of length and width of 20 basidiospores. The identified specimens have been deposited in the Herbarium of Botany Department (PUN), Punjabi University, Patiala, India under the voucher numbers PUN5535 and PUN5537.

Molecular characterization: Genomic DNA from basidiocarp cap and pure culture was extracted according to Zhou et al. (1999) and quantified with a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). The ITS region of nrRNA was amplified by polymerase chain reaction (PCR) with ITS1 and ITS4 primers (White et al. 1990) using a PCR Thermal Cycler (Perkin Elmer, USA). PCR amplification was carried out in a volume of 50 µl containing 100 ng genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, and 2.5 units of *i*-taq™ DNA polymerase (iNtRON Biotechnology, KOREA). The thermal cycling conditions were initial denaturation for 3 min at 95°C, 34 cycles of 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C followed by a final extension of 7 min at 72°C. Before sequencing, PCR products were purified with Qiaquick columns (Qiagen) and subcloned using InsTAclone, PCR cloning kit (Thermo Scientific), as per the manufacturer's instructions. Subsequently, the *Escherichia coli* DH5α cells were transformed and the clones containing the ITS insert were selected randomly and sequenced. The representative sequences for basidiocarp and culture have been deposited in the GenBank under the accession numbers KF699850 and KJ472765, respectively.

Phylogenetic analysis: A nucleotide BLAST search was performed to find the possible sister groups of the newly sequenced taxa. Sequences of closely related species were retrieved from the

GenBank including the sequences of *S. sibiricus* reported from the Himalayan region by Sarwar et al. (2011). In case of large number of homologous sequences for a particular species, only a subset was selected. The sequences were aligned using MAFFT ver. 7.0 (Katoh & Standley 2013) along with our sequences and edited with BioEdit 5.0.6 (Hall 1999). The aligned data set was deposited in TreeBASE (14778). Bayesian analysis was conducted with MrBayes 3.2.1 (Ronquist et al. 2012) with two simultaneous independent runs of four chains (one cold and three heated) each. The analysis was run using Metropolis-coupled Markov Chains Monte Carlo search algorithm until the chains converged in two simultaneous runs, after 2,000,000 generations. The sample frequency was set to 100, resulting in total of 20,000 trees and the first 5,000 trees were discarded as "burn in" (0.25). Bayesian posterior probabilities were calculated from the 50% majority rule consensus of the remaining 15,000 trees.

Results

Molecular phylogeny: The PCR products amplified with ITS1 and ITS4 were 693 bp in length and BLASTn search showed maximum similarity (99% for 98% query coverage) with *S. sibiricus* strain HMAS 6601 (Wu et al. 2000). The ITS matrix consisted of 47 taxa representing homologous ITS sequences of 12 closely related *Suillus* spp., and *Rhizopogon subcaerulescens* as an out-group taxon. The aligned dataset contained 691 characters including gaps. Bayesian analysis of the ITS region yielded a consensus tree shown in Fig. 3. Bayesian consensus showed splits between *Suillus himalayensis* and a well-resolved clade containing *S. americanus* (Peck) Snell, which is supported by posterior probability percentage.

Taxonomy

Suillus himalayensis B.Verma & M.S.Reddy, **sp. nov.** Figs 1 & 2

MycoBank no.: MB 805647

Etymology: The specific epithet "himalayensis" refers to the new *Suillus* species being reported from the Himalayan region of India.

Diagnosis: The fungus resembles *Suillus americanus* (Peck) Snell, but differs morphologically by the yellow white to pale yellow pileus with grayish greenish tinge, brown colored appressed fibrillose scales, white colored pileal veil, and the pileal context changing to grayish green and tubes to olive brown on bruising. Microscopically, more cylindrical spores, larger basidia, and less broaden cystidial elements distinguish it from *Suillus americanus*.

Detailed description

Macrocharacters: Basidiocarps 3.0–11.5 cm in height. Pileus 3.5–8.0 cm broad, convex when young, flattened with age; margin irregular and slightly upturned; pileal veil white ($1A_1$), cottony, collapsing with age and finally evanescent; pileus surface moist to viscid, yellowish white ($1A_2$) to pale yellow ($1A_3$) with grayish green ($1C_3$–$1C_5$) tinge, cuticle fully peeling, umbo absent; scales brown colored appressed fibrillose throughout the surface, more prominent on the margins. Pileus context 5–15 mm thick, pale yellow ($1A_3$), turning grayish green on bruising; odor not distinctive and

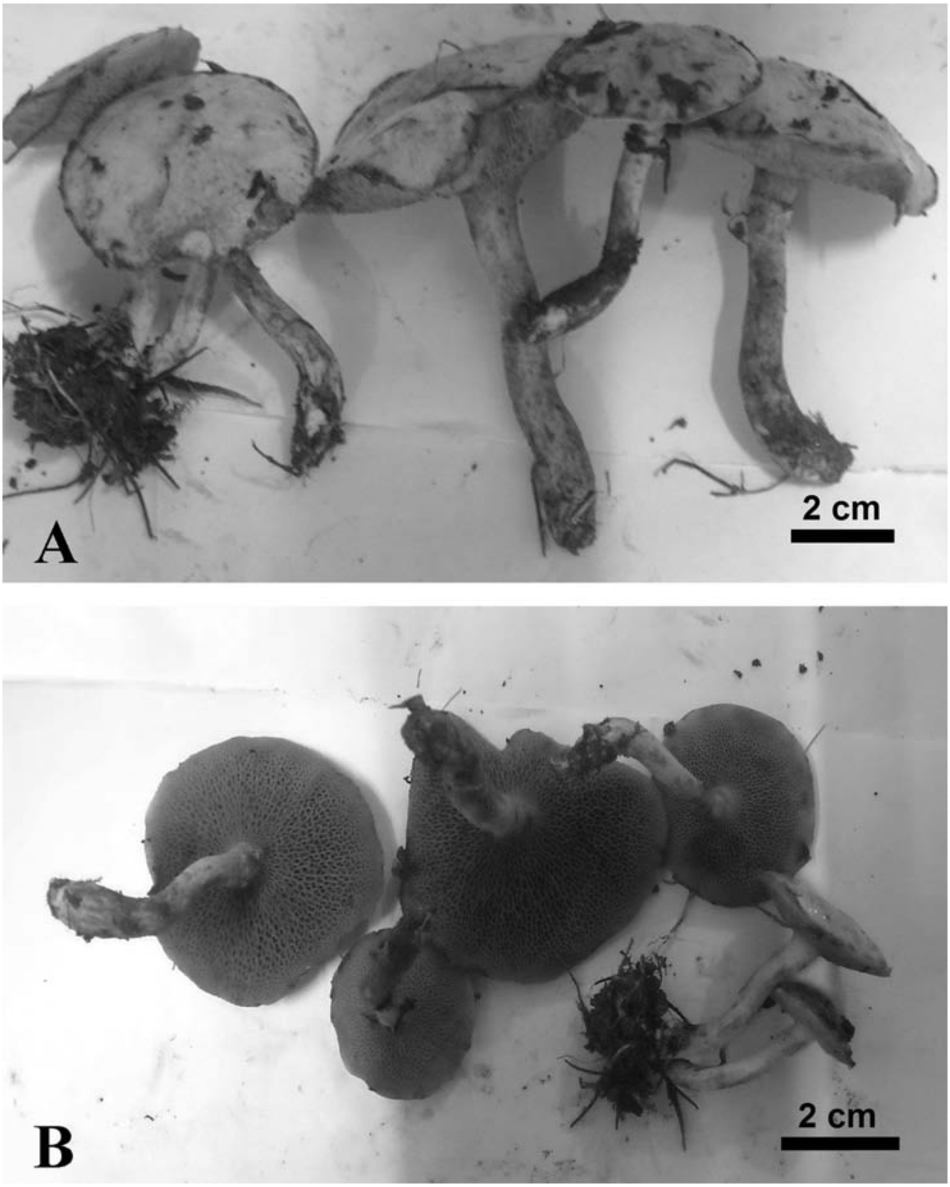

Fig. 1. *Suillus himalayensis* basidiocarps (PUN 5535, holotype). A. Basidiocarps showing light greenish tinge and brownish squamules over the pileal surface; B. Basidiocarps showing tubes, stipe, and veil remnants attached to the stipe

taste mild. Tubes 5–7 mm deep, decurrent, radial, crowded, fully peeling, pale yellow (2A$_3$) when young, grayish yellow (2C$_4$–2C$_5$) with age, slowly staining (3–5 min) to

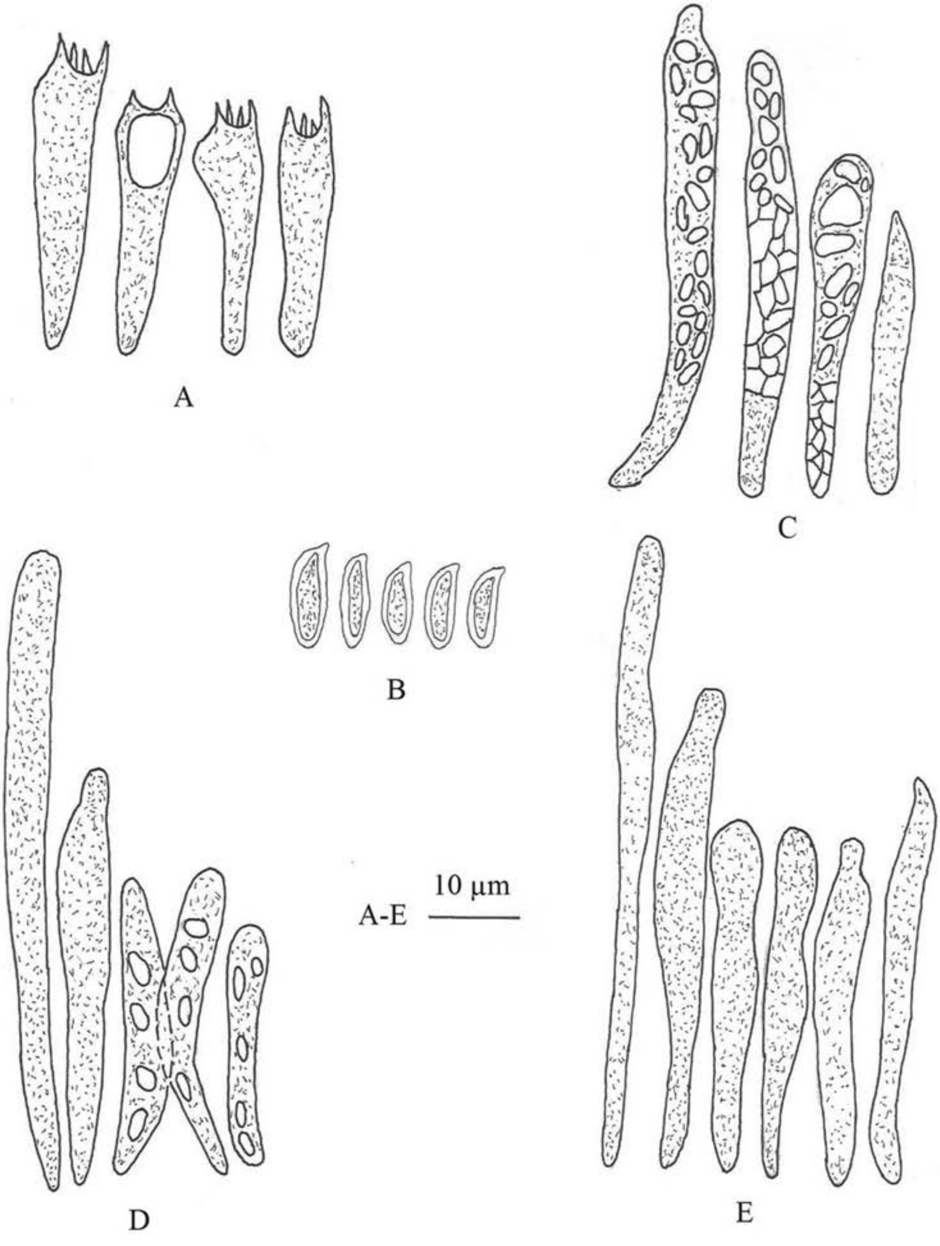

Fig. 2. Microscopic characters of *Suillus himalayensis* (PUN 5535, holotype). A. Basidia; B. Basidiospores; C. Pleurocystidia; D. Cheilocystidia; E. Caulocystidia

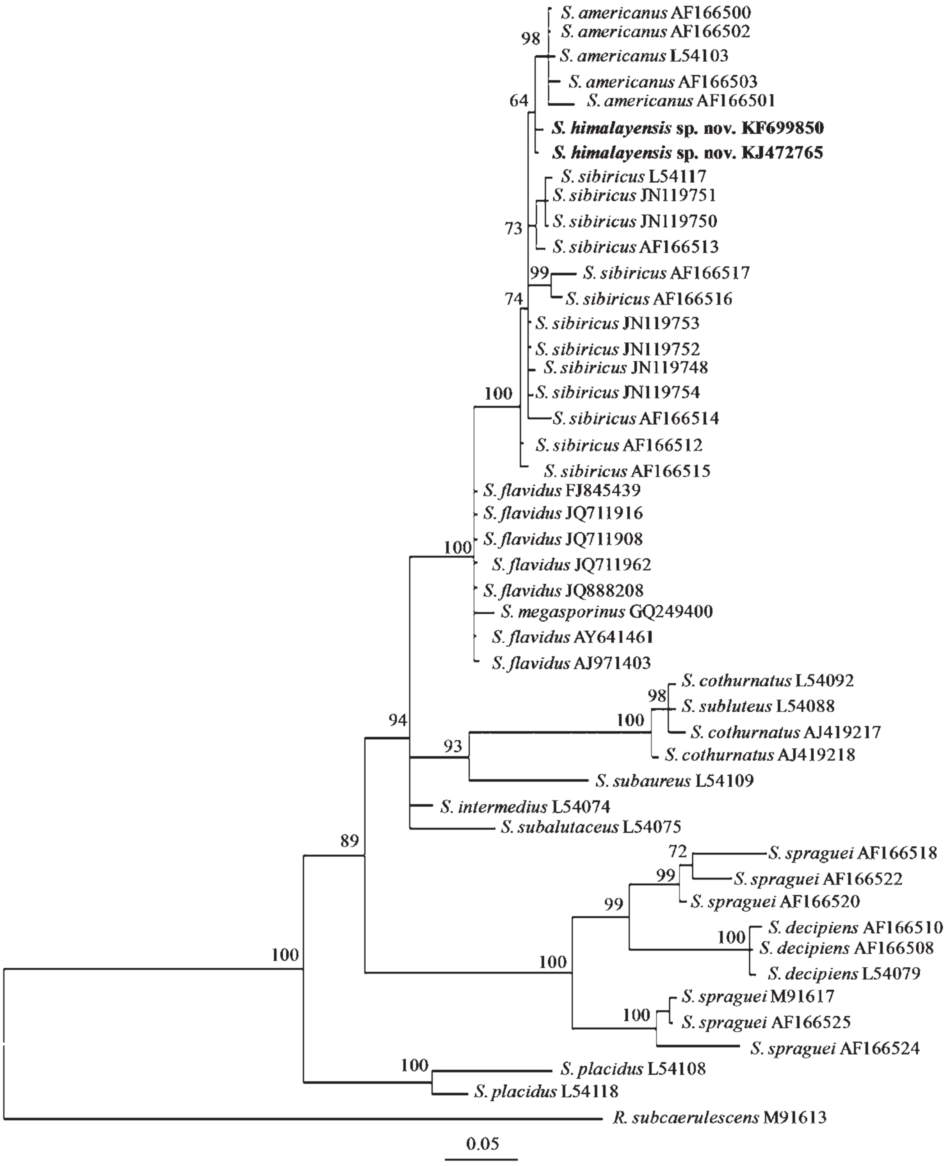

Fig. 3. Phylogenetic tree of *Suillus himalayensis* and related species inferred from bayesian analysis of the ITS region with *Rhizopogon subcaerulescens* as an out-group taxon. Bayesian posterior probability percentages are indicated at the nodes of the tree.

olive brown on bruising; pore mouths angular, large, 1–2 mm broad. Stipe central to excentric, 2.8–8.5 cm long, 4–12 mm thick, tubular and equal in diameter, covered with brown black glandular dots throughout, color yellow ($2A_5$) at top, pale yellow ($2A_3$) in middle and white ($1A_1$) at base, grayish yellow to olive brown when handled; annulus lacking, veil remnants attached to stipe in rare specimens; mycelium at base white. Stipe context solid, pale yellow ($1A_3$), and changing grayish green when bruised.

MICROCHARACTERS: Spore deposit light brown ($6D_8$), spores 9–12 × 3.0–3.8 µm (Q=3.0), cylindrical, hyaline to brownish in KOH, yellowish in Melzer's, smooth, and granular content inside. Basidia 22–30 × 6–8 µm, clavate, granular, 4 spored, occasionally 2 spored, hyaline to yellowish in KOH, and yellowish in Melzer's; sterigmata 1.5–4.6 µm high. Pleurocystidia 30–55 × 4.6–6.2 µm, cylindrical to subclavate with acute, capitate to blunt tips, granular mostly with brown coagulated contents in cystidia, in fasicles or scattered, hyaline to slightly brownish in KOH, and yellowish brown in Melzer's. Cheilocystidia 26–70 × 3.8–6.2 µm, abundant, mostly in fascicles, and almost similar to pleurocystidia. Caulocystidia at apex 38–70 × 4.6–6.2 µm, cylindrical to subclavate with acute, capitate and blunt tips, granular without any brown contents, and absent at base. Trama divergent. Clamp connections absent.

CHEMICAL COLOR REACTIONS: Pileal flesh: 2.5% KOH – reddish, 10% $FeSO_4$ – olive, 14% ammonia – reddish brown then to black, conc. HNO_3 – no color reaction. Pileus cuticle: 2.5% KOH – light brown then to blue black, 10% $FeSO_4$ – blackish brown, 14% ammonia – brown then to black brown, conc. HNO_3 – no color reaction.

COLLECTIONS EXAMINED: INDIA. Himachal Pradesh: Mandi: Barot (1829 m); Lachkandi, 7th August 2011, PUN5535 (Holotype); Tikkan (5 km from Lachkandi), 28th July 2012, PUN5537 (Paratype).

HABITAT: Solitary to scattered on humicolous soil under young to moderate aged *Pinus wallichiana* trees.

Discussion

Macro and microscopic characters of the present species have been compared with the species described in Smith and Thiers (1964, 1971), Corner (1972), Thiers (1979) and Sarwar et al. (2011). Morphologically, the species come close to *S. americanus* (Peck) Snell and *S. sibiricus* (Singer) Singer. Till date, there is no record for *S. americanus* from India and *S. sibiricus* is the abundantly fruiting and most dominating *Suillus* species associated with *P. wallichiana* in the northwestern Himalayas. We have compared the present specimens with both these species (Table 1).

Morphologically as well as molecularly, *S. americanus* is the nearest relative of *S. himalayensis*. The species varies in possessing the yellow white to pale yellow pileus with light greenish tinge, brownish squamules, white pileal veil, upturned margin and plane apex as compared to the bright yellow pileus surface, orange to reddish squamules, yellowish pileal veil, incurved margin and occasional presence of low obtuse umbo in *S. americanus*. Also, the species differs as its flesh changes to grayish green and tubes to olive brown color on bruising in contrast to vinaceous brown in *S. americanus*. Microscopically, the less broaden cystidial elements (3.8–6.2 µm) than *S. americanus* (7–12 µm) is the main distinguishing characteristic of the present specimens. Basidia are larger and basidiospores more cylindrical (Q=3.0) than *S. americanus* (Q=2.5).

Table 1. Comparative macro and microscopic characters of *Suillus himalayensis* sp. nov. with *S. americanus* and *S. sibiricus* species.

Name of species:	***Suillus himalayensis* sp. nov.**	***Suillus americanus***	***Suillus sibiricus***
Pileal color	Yellow white to pale yellow with light green tinge	Bright yellow	Dingy olive yellow
Squamules	Brownish	Orange to reddish	Brownish
Pileal veil	White	Yellowish	White to dingy yellow
Context color change on bruising	Grayish green	Vinaceous brown	Dull cinnamon
Tubes color change on bruising	Olive brown	Vinaceous brown	Dull cinnamon
Tubes size	5–7 mm	4–6 mm	5–15 mm
Umbo	Absent	Plane or with a low obtuse umbo	Plane or slightly umbonate
Pleurocystidia	30–55 × 4.6–6.2 µm	38–60 × 7–11 µm	40–70 × 6–9 µm
Basidia	22–30 × 6–8 µm	17–22 × 5–7 µm	22–28 × 5–7 µm
Basidiospores			
Range	9–12 × 3–3.8 µm	7–11 × 3–4.8 µm	7–12 × 3.2–4.8 µm
Mean L; W; Q	10.3; 3.4; 3.0	9.5; 3.7; 2.5	9.8; 4.1; 2.4

L=length, W=width, Q=length/width.

The second nearest species is *S. sibiricus*, but it differs from that of *S. himalayensis* by the pileus rather dingy olive yellow, flesh and tubes changing to dull cinnamon on bruising, tubes 5–15 mm, slight umbo present occasionally, cystidial elements broader (6–9 µm), and the basidiospores being less cylindrical (Q=2.4).

The phylogenetic results show that *S. himalayensis* forms a separate clade when compared with the ITS sequences of other *Suillus* spp. Posterior probability value supports the species as a different clade in the group. These findings show that *S. himalayensis* is clearly distinct from other closely related species of *Suillus* and has its close ancestry to *S. americanus*. Based on morphological characters as well as the molecular analysis of the ITS region, a new *Suillus* species *Suillus himalayensis* has been proposed.

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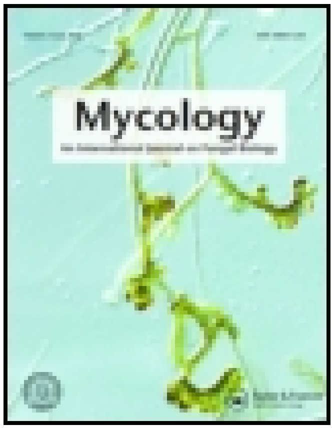

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Suillus indicus sp. nov. (Boletales, Basidiomycota), a new boletoid fungus from northwestern Himalayas, India

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Suillus indicus sp. nov. (Boletales, Basidiomycota), a new boletoid fungus from northwestern Himalayas, India

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The new species *Suillus indicus* is described based on the morpho-anatomical description and molecular analysis of basidiomes found in conifer forests of the northwestern Himalayas, India. Morphologically, the key diagnostic characteristics of the new taxon are brownish-orange to reddish-brown pileus with low obtuse umbo, brownish-red to reddish-brown fibrillose squamules over the pileal surface, and absence of fibrillose squamules and glandular dots on the stipe surface. Sequences derived from the internal transcribed spacer region of basidiomes and culture demonstrated that the species is clearly distinct from other known taxa of *Suillus* and new to science.

Keywords: *Suillaceae*; ectomycorrhizal; ITS; phylogeny; taxonomy

Introduction

The genus *Suillus* Gray is one of the most prominent genera of ectomycorrhizal fungi in the order *Boletales* with about 50 species described worldwide (Kirk et al. 2008). *Suillus* species are confined to the temperate, boreal, and Mediterranean regions, although there are few reports (Natarajan & Raman 1983; Halling & Mueller 2002) of their occurrence from tropical areas. Most of *Suillus* species known so far are documented from the northern hemisphere, but some have also been reported from southern hemisphere (McNabb 1968; Watling & Gregory 1989; Dunstan et al. 1998) associated with the introduced pine species. *Suillus* species exhibit narrow host range forming ectomycorrhizae mainly with the members of the family *Pinaceae* and also with some deciduous species (Singer 1986; Kretzer et al. 1996; Wu et al. 2000). Based on the internal transcribed spacer (ITS) sequence analysis, Kretzer et al. (1996) have demonstrated that in the genus *Suillus, Larix* association seems to be primitive and associations with pines, Douglas-fir, and hardwoods seem to be derived in the genus *Suillus*.

The present knowledge of *Suillus* diversity from India, particularly from northwestern Himalayan region, is scanty. *Suillus brevipes* (Peck) Kuntze, *S. pallidiceps* A. H. Sm. and Thiers, *S. punctatipes* (Snell and E.A. Dick) Singer and *S. subluteus* (Peck) Snell have been reported from *Pinus patula* Schltdl. and Cham. forests of Tamilnadu, South India (Natarajan & Raman 1983). *Suillus sibiricus* (Singer) Singer and *S. granulatus* (L.) Roussel have been shown to be ectomycorrhizal with *Pinus wallichiana* A.B. Jacks. (Sagar & Lakhanpal 2005; Dar et al. 2010), whereas *S. triacicularis* Verma and Reddy has been described fruiting exclusively in association with *Pinus roxburghii* Sarg. (Verma & Reddy 2014) from the northwestern Himalayan region of India. In the last few years, we have conducted extensive surveys to the conifer forests of this region with the aim to document and preserve the diversity of *Suillus* and detect some new species of this genus. During our collection trips to northwestern Himalayas, *Suillus* species were found mostly in early monsoon season and *Suillus sibiricus* was observed to be the most frequently encountered and widely distributed *Suillus* species. During our expeditions to Himachal Pradesh in 2010 and 2011, two different specimens of a putative new species showing close morphological resemblance to *S. decipiens* (Peck) Kuntze were collected from mixed conifer forests. In the present study, morphological and molecular data obtained for these specimens are used to clarify the systematic position of the new species *Suillus indicus* within the genus.

Materials and methods

Collection sites and fungal isolation

Reference material for the basidiomes has been deposited in the Herbarium of Botany Department (PUN), Punjabi University, Patiala, India, under the voucher numbers PUN 6576 and PUN 6578. Fresh basidiomes of *S. indicus*

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were collected from Narkanda (latitude 31°27′N and longitude 77°45′E) and Kandyali (latitude 31°13′29″N and longitude 77°26′4″E) sub localities of Shimla, Himachal Pradesh, India. The vegetation of the collection sites is dominated by randomly distributed pure and mixed stands of *P. wallichiana* and *Cedrus deodara* (Roxb. ex D. Don) G. Don.

Malt Extract (ME) agar (2% w/v) media supplemented with streptomycin (50 µg/ml) was used to isolate pure culture. Surface of fresh basidiomes collected was sterilized with rectified spirit and cut along the pileal surface with sterile surgical blades to expose the inner pileal context. Two to three pieces of clean fresh pileal context 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 culture isolated for *S. indicus* has been designated as "Isolate SNW02" and submitted to Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India, under the accession number MTCC 11955.

Morphological description of basidiomes

Standard methodology and terminology used for describing the basidiomes followed Corner (1972). Macro-morphological description, macro-chemical reactions, habitat, and plant association are based on detailed field notes of fresh basidiomes. Color codes in the macroscopic descriptions are from Kornerup and Wanscher (1978). Anatomical features were observed from dried material by reviving the sections either in water or in 3% potassium hydroxide (KOH). Measurements were made at 1000× magnification with a calibrated ocular micrometer on an Olympus light microscope (Olympus, Tokyo, Japan) by mounting the preparations in lactophenol cotton blue. Basidiospores and basidia were measured from the hymenophore of mature basidiomes. The spore measurements exclude the length of apiculus and the basidium length excludes the length of sterigmata. Quotient value ($Q = L/W$) was calculated considering the mean value of length and width of 20 basidiospores. Microscopic line drawings of microstructures were made from rehydrated material with the aid of a camera lucida.

Molecular characterization

For phylogenetic analysis of *S. indicus*, genomic DNA from dried basidiome and culture was extracted based on Zhou et al. (1999). DNA extracts were then quantified with a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA) and stored at −20°C until use. The ITS region of nrRNA was amplified with the universal primers ITS1 and ITS4 (White et al. 1990) using a polymerase chain reaction (PCR) Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions applied for the ITS region included an initial denaturation for 5 min at 94°C followed by 34 cycles of 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C and a final extension of 7 min at 72°C. PCR products were purified with Qiaquick columns (Qiagen, Hilden, Germany), following the manufacturer's instructions and sequenced. The ITS sequences obtained from isolate SNW02 and basidiome of *S. indicus* have been deposited in the GenBank under the accession numbers KJ675500 and KJ675502, respectively.

Phylogenetic analysis

The ITS sequences acquired in this study were compared with those available in the GenBank database by using the BLASTn search algorithm. To find out the possible sister groups for newly sequenced taxon, a preliminary phylogenetic analysis was performed using MrBayes v.3.2.2 (http://mrbayes.sourceforge.net/; Ronquist et al. 2012) by considering the ITS sequences representing all *Suillus* species as mentioned by Bruns et al. (2010). On the basis of preliminary analysis, a group comprising 27 *Suillus* species including our taxon was selected for phylogenetic analysis. Sixty five ITS sequences for other 26 *Suillus* species were retrieved from GenBank. *Suillus triacicularis* (KF977189), which phylogenetically belongs to the *S. granulatus* group (Bruns et al. 2010; Verma & Reddy 2014), was taken as an outgroup taxon. Alignment of the sequences was constructed using MAFFT ver. 7.0 (http://mafft.cbrc.jp/alignment/server/; Katoh & Standley 2013) and edited with BioEdit 5.0.6 (http://www.mbio.ncsu.edu/bioedit/; Hall 1999). The aligned data set has been deposited in TreeBASE (15673). Phylogenetic analysis on the resulting alignment was performed using Bayesian Inference (BI). A Bayesian analysis was implemented in MrBayes v.3.2.2 with two parallel runs each one consisting of four incrementally heated Monte Carlo Markov Chains. The analysis was run using Metropolis-coupled Markov Chains Monte Carlo search algorithm over 2,000,000 generations and the convergence of Bayesian analysis was observed by examination of the standard deviation of split frequencies <0.01. Trees were sampled every 100th generations resulting in total of 20,000 trees. The first 5000 trees, representing the burn-in phase of the analysis, were discarded and the remaining 15,000 trees were used to calculate posterior probabilities (PP) from the 50% majority rule consensus trees.

Results

Phylogenetic inference

The PCR products amplified with ITS1 and ITS4 were 702 bp in length. Sequence analysis by BLAST revealed 93% similarity (query coverage of 99%) with unidentified

Suillus sp. K91S8 (GQ267488) from New Zealand (Walbert et al. 2010) and 92% similarity (99% query coverage) with *S. flavidus* isolate FFP962 (JQ711908) from Canada (Jones et al. 2012). The alignment of ITS sequences of selected *Suillus* species resulted in a data matrix comprising 28 taxa and 713 characters including gaps. Bayesian analysis of ITS region for the selected *Suillus* group yielded a consensus tree (Figure 1) and divided the species broadly into five major clades (*Suillus*I, *Suillus*II, *Suillus*III, *Suillus*IV, and *Suillus*V). The clade *Suillus*V consisted of two ITS sequences of the present study (*S. indicus*) and forms an independent clade in the group, which is well supported by the Bayesian posterior probability percentage (100%). The ITS sequences of *Suillus spraguei* (Berk. and Curt.) Kuntze derived from American (M91617, AF166524, and AF166525) and Chinese collections (AF166518, AF166520, and AF166522) are paraphyletic and subdivided into two different subclades. Chinese *S. spraguei* isolates were found to be sister to American *S. decipiens* isolates (L54079, AF166508, and AF166510) rather than American *S. spraguei* isolates. Contrastingly, a few pairs of *Suillus* species (*S. flavidus* and *S. megaporinus, S. cothurnatus* and *S. subluteus, S. laricinus* and *S. grisellus*) are not distinguished by the ITS locus.

Taxonomy

Suillus indicus B. Verma and M.S. Reddy, **sp. nov.** (Figures 2 and 3). MycoBank No.: MB 808527.

Basidiomes: ranges 4.0–10.0 cm in height. ***Pileus***: 3.0–9.0 cm broad, convex when young, flattened or slightly upturned with age; margin regular, appendiculate with white velar fragments (1A1); pileus surface dry to moist, not viscid or slightly viscid only when wet, brownish-orange (6C4–6C6) when young, reddish-brown (8D7–8D8) in age, ground color discoloring to pale yellow with age, low obtuse umbo present and cuticle fully peeling; brownish-red (8C7–8C8) to reddish-brown (8D7–8D8) fibrillose squamules present throughout the pileal surface. Pileus context ≤20 mm thick, soft, pale yellow (2A3) to yellowish (2A6) in age, unchanging or slowly staining pinkish when exposed; taste mild and odor not distinctive. ***Tubes***: up to 8 mm deep, decurrent, radially arranged, fully peeling, yellow

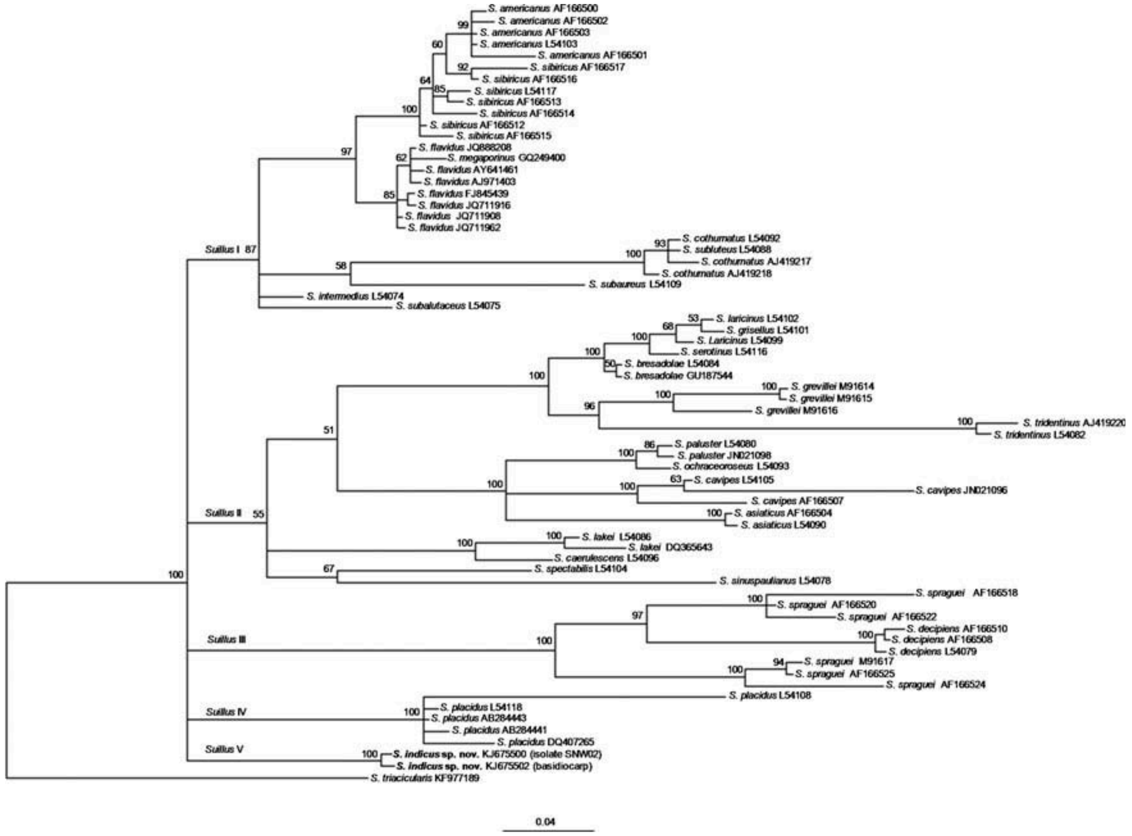

Figure 1. Bayesian tree showing the relationships between the internal transcribed spacer (ITS) sequences derived from the basidiome and isolate SNW02 (indicated in the brackets) of *Suillus indicus* (shown in bold) and those of related species retrieved from GenBank. Numbers at nodes stand for the posterior probability percentages (>50%) of the Bayesian analysis (outgroup: *Suillus triacicularis*).

Figure 2. *Suillus indicus* basidiomes: A. Young basidiome showing umbo and very few appressed fibrillose squamules on the pileus; B. Young basidiome showing white partial veil and absence of glandular dots on the stipe surface; C. Mature basidiome with appressed fibrillose squamules and a low obtuse umbo on pileal surface; D. Stipe with annulus and no glandular dots/smears. Scale bars: A–B = 1 cm, C–D = 2 cm.

(3A6–3A7) when young, brownish-yellow (5C7–5C8) with age, slowly turning light brown on bruising; pore mouths irregular, 1–3 mm broad. ***Stipe***: central, 3.0–8.0 cm long, 1.0–1.2 cm thick, cylindrical and slightly enlarged downward, typically clavate in young specimens, glandular dots absent, concolorous with the tubes but white (1A1) at base; white colored annulus present turning brown with time; basal mycelium at base white. Stipe context solid, pale yellow (2A3–2A6) and turning dirty brown when exposed. ***Basidiospores***: cinnamon brown (6D6) in mass, hyaline to pale yellow in KOH, yellowish in Melzer's reagent, smooth with granular content inside, 7.5–11.5 × 3.0–4.5 µm in size and cylindric to subcylindric in shape (Q = 2.6). ***Basidia***: 17.0–30.0 × 4.5–6.5 µm, clavate, 4-spored, occasionally 2-spored, hyaline to yellowish in KOH, yellowish with yellowish brown contents in Melzer's reagent; sterigmata 1.5–3.1 µm high. ***Pleurocystidia***: 18.0–54.0 × 4.0–8.5 µm, cylindrical to subclavate with blunt to constricted tips, scattered or in fascicles, mostly with brown coagulated granular contents inside, hyaline in KOH, yellowish brown in Melzer's reagent. ***Cheilocystidia***: 20.0–62.0 × 3.0–5.0 µm, shape similar to pleurocystidia, granular, slightly yellowish in KOH, brownish in Melzer's reagent, abundant and mostly in fascicles. ***Caulocystidia***: absent. Trama divergent. Clamp connection absent.

Chemical color reactions

Pileal context: 2.5% KOH – pink to bluish gray, 10% $FeSO_4$ – olive gray, 14% ammonia – pinkish red to bluish gray, concentrated HNO_3 – no color reaction. Pileus cuticle: 2.5% KOH – greenish-black, 10% $FeSO_4$ – blue-black, 14% ammonia – dark green to blue-black, concentrated HNO_3 – no color reaction. Baroni (1978) studied the chemical spot tests of 6 genera of *Boletaceae* and found that pink to reddish color reaction of pileal context with KOH and ammonia distinguishes *Suillus* species from other *Boletaceae*.

Habitat

Solitary on humose soil under *Cedrus deodara* trees in mixed forest of *Pinus wallichiana* and *C. deodara*.

Specimens examined

INDIA, Himachal Pradesh, Shimla: Narkanda, 2621 m, 27 July 2010, growing solitary on humicolous soil in mixed forest of *P. wallichiana* and *C. deodara, B. Verma* (Holotype: PUN 6576); Kandyali, 2450 m, 29 July 2011, growing solitary on soil in mixed forest of *P. wallichiana* and *C. deodara, B. Verma* (Paratype: PUN 6578).

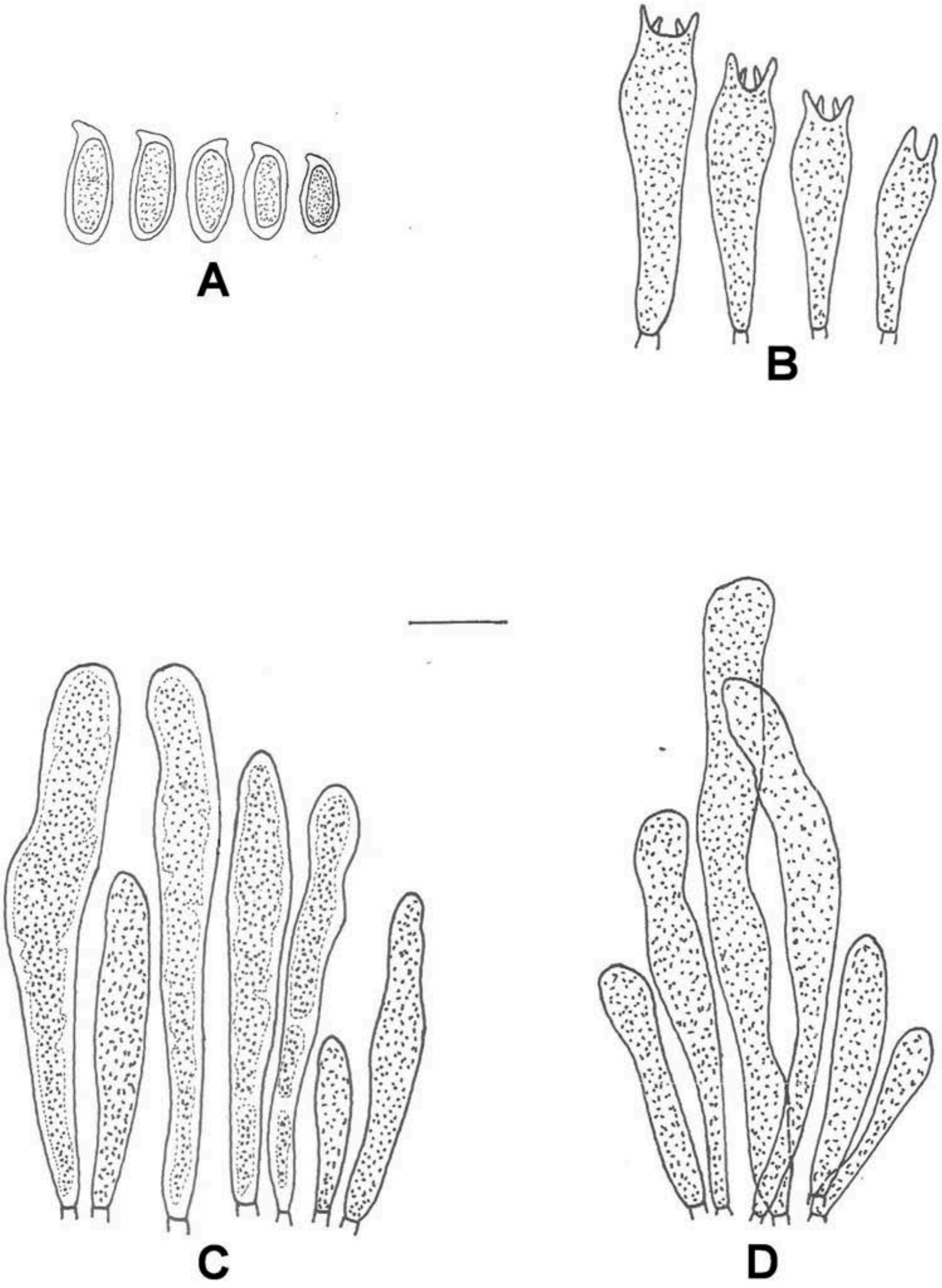

Figure 3. Microscopic line drawings of *Suillus indicus* (holotype): A. Basidiospores; B. Basidia; C. Pleurocystidia; D. Cheilocystidia. Scale bar: A–D = 10 µm.

Commentary

The species is morphologically close to *Suillus decipiens* (Peck) Kuntze, but can be distinguished mainly by the absence of fibrillose squamules over the stipe, umbonate pileus and less numerous fibrillose squamules over the pileal surface. Microscopically, the species differs from *S. decipiens* by the presence of few 2-spored basidia on hymenium and absence of caulocystidia on stipe surface.

Etymology

The specific epithet *indicus* is taken from Latin with reference to the new species being reported from the Indian region.

Discussion

In the Bayesian analysis of the *Suillus* group, selected for the phylogenetic inference in the present study, a few pairs of *Suillus* species (*S. flavidus* and *S. megaporinus, S. cothurnatus* and *S. subluteus, S. laricinus* and *S. grisellus*) are not distinguished by the ITS locus suggesting that there are limitations of using ITS locus for species-level identifications in the genus *Suillus*. This might be due to over-description (=synonymy) or the lack of ITS divergence between the sibling species (Bruns et al. 2010; Verma & Reddy 2014). The *S. spraguei* isolates (American and Chinese) analyzed showed remarkable heterogeneity and were divided into two different subclades. Chinese *S. spraguei* clade is sister to *S. decipiens*, whereas American *S. spraguei* is paraphyletic. On the basis of ITS data, Wu et al. (2000) also observed similar heterogeneity among the Chinese and American *S. spraguei* isolates.

The phylogenetic analysis of selected *Suillus* taxa clustered them mainly into five different clades (Figure 1). According to Smith and Thiers (1964) and Klofac (2013), these clades can be morphologically distinguished on the basis of few basic morphological characteristics such as presence or absence of veil, annulus, and glandular dots on the basidiomes. All of the clades, except *Suillus*IV, mainly comprise species exhibiting a well-developed veil or a false veil in young specimens either leaving an annular zone or a true annulus on the stipe or otherwise adhered to the pileal margin at maturity. On the other hand, the clade *Suillus*IV consists of a single species *S. placidus* (Bonord.) Singer, which has no veil and annulus at any stage of the development but glandular dots or smears are present on the stipe (Singer 1945; Smith & Thiers 1964). The first four clades comprising *Suillus* species with a well-developed veil or false veil in young specimens can be further divided on the basis of presence or absence of glandular dots on the stipe. The clade *Suillus*I mainly comprises species with glandular dots, while the clades *Suillus*II, *Suillus*III, and *Suillus*V comprise only species that lack glandular dots on the stipe. Furthermore, the species in clades *Suillus*II, *Suillus*III, and *Suillus*V can be classified on the basis of plant host to which they are found to be associated in nature. All the *Suillus* species in clade *Suillus*II are generally associated either with *Larix, Pseudotsuga, Abies,* or *Picea* (Smith & Thiers 1964; Klofac 2013) as compared to the species in clade *Suillus*III (Kuntze 1898; Smith & Thiers 1964; Wu et al. 2000; Burchhardt et al. 2011; Klofac 2013), which are found to be associated only with *Pinus* species. *Suillus indicus* specimens (clade *Suillus*V) were collected from mixed forests of *P. wallichiana* and *C. deodara* under *C. deodara* trees suggesting its probable association with *C. deodara*, although its association with *P. wallichiana* cannot be denied. In fact, host shifts of basidiomycetes are considered to be major driving forces in the evolution process (Refrégier et al. 2008; Li et al. 2009, 2011; Rochet et al. 2011). Thus, the *Suillus* species of selected group are phylogenetically grouped on the basis of their basic morphological features and the host specificity. The Bayesian analysis clustered *S. indicus* as a distinct clade (*Suillus*V) in the group distinguishing it from all other *Suillus* species.

Morphologically, *S. indicus* come close to *S. decipiens* but differs considerably identifying it as a distinct species. The presence of umbo, less numerous/prominent fibrillose squamules over the pileal surface and absence of squamules over the stipe differentiate it from *S. decipiens*. Anatomically, the occasional presence of two-spored basidia and absence of caulocystidia distinguish the species from *S. decipiens*. *Suillus spraguei* is the next closest species, which is commonly referred to as *Suillus pictus* A.H. Sm. and Thiers although the name *S. spraguei* is used for one or even several disjunct populations of *S. pictus* in Asia (Wu et al. 2000; Burchhardt et al. 2011; Klofac 2013). The pileus color of young and fresh *S. spraguei* specimens is much redder than *S. decipiens* and *S. indicus* but the faded specimens strongly resemble *S. decipiens*. Furthermore, in contrast to *S. indicus, S. spraguei* also bears fibrillose squamules on the stipe. The combination of morpho-anatomical features and phylogenetic analysis of ITS sequences derived from fruiting bodies and culture distinguishes *S. indicus* as a distinct species and we herein report it as new to science.

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