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 4ºC 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^{o}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^{o}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

$$\text{CEC}\left(\frac{\text{cmol}}{\text{kg}}\right) = \frac{\text{mg NH}_4 \text{ in extract} - \text{mg NH}_4 \text{ in blank}}{18}$$

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

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

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^{o}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^{o}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^{o}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.