

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**

ACKNOWLEDGEMENTS

This thesis was made possible by the financial support provided by University Grant Commission (UGC), Bahadur Shah Zafar Marg, New Delhi, India and the research facilities provided by TIFAC-Core, Thapar University, Patiala, Punjab, India.

Foremost, I wish to express my intense gratitude to my supervisor Professor **M. Sudhakara Reddy**, Department of Biotechnology, Thapar University, Patiala for giving me the opportunity to work with ectomycorrhizal fungi. His inspiring attitude and broad knowledge have helped me throughout my thesis work. I am very grateful for all his help, support, positive feedback, critical guidance and constructive criticism from the inception of my work to the final manuscript.

I extend my sincere thanks to Professor Dinesh Goyal, Head, Department of Biotechnology, Thapar University, Patiala for providing pleasant environment and good working facilities during my thesis work.

I am most grateful to Professor N. S. Atri, Department of Botany, Punjabi University, Patiala for his training, guidance and moral support. Without his efforts it would not have been possible for me to accomplish the taxonomic part of this thesis.

I express my warmest thanks to Professor T. D. Bruns, Department of Plant and Microbial Biology, University of California, Berkeley, California and Mrs. Rui Zhang, Plant Biology and Conservation, Northwestern University, Chicago for providing literature, valuable discussions and general support during my research work.

The members of my committee, Dr. N. Tejo Prakash and Dr. Ranjana Prakash, were always concerned, encouraging, patient and extremely helpful throughout my academic pursuit. I am immensely grateful to both of them.

This work could not have been completed without the assistance, technical support and help provided by Mr. Lallan Yadav and Mr. Babban Yadav during the research development. I express my sincere thanks for their efforts.

Amongst my fellows, I am very grateful for all the hope, cheer and suggestions that I received from Dr. Raghavendra Aminedi, Dr. Diwakar Aggarwal, Dr. Kunal, Dr. Gurdeep Kaur and Dr. A. Giridhar Babu.

I want to cordially thank my wife Mrs. Samidha Sharma for her personal support and great patience at all times. She always kept me on track and provided encouragement at each and every moment of my thesis work.

As usual, my parents (Sh. Virender Verma and Smt. Geeta Devi) have given me their unambiguous support throughout, for which my mere expression of thanks likewise does not suffice. It is inevitable to recognize the kind co-operation and help; I received from my sisters, Mrs. Poonam Verma and Mrs. Kalpna Verma, and from my brother-in-law, Mr. Amit Verma.

I want to thank all my relatives and friends for their love and support. I wish to express my warmest thanks to my mother-in-law Mrs. Arti Sharma for general support and taking care of our baby whenever needed.

Above all, I would like to thank the almighty God for blessing me with a little angel “**Deehar**” with whom I have spent and still enjoying a wonderful time.

Finally, I would like to sincerely thank each and everyone who supported and assisted me on this project and for whom it is not possible to mention over here.



(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](https://doi.org/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.

TABLE OF CONTENTS

Contents	Page No.
Acknowledgements	iv
List of Publications	vi
Abstract	vii
Table of Contents	viii
List of Figures	ix
List of Tables	xiii
Abbreviations	xv
1. Introduction	1-11
2. Review of literature	12-47
3. Materials and methods	48-94
4. Results	95-167
5. Discussion	168-191
6. Summary	192-197
References	198
Appendix I	254
Appendix II	259

List of Figures

Figure 1.1	Schematic diagram showing difference between ectomycorrhizae and endomycorrhizae colonization of plant roots	3
Figure 2.1	Diagrammatic representation of fungal ribosomal DNA (rDNA) repeat unit	20
Figure 3.1	Map of northwestern Himalayan terrain of India, showing different districts of three states (Jammu and Kashmir, Himachal Pradesh and Uttarakhand) from where <i>Suillus</i> specimens were collected	49
Figure 4.1	<i>Suillus triacicularis</i> (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	99
Figure 4.2	<i>Suillus triacicularis</i> , microscopic line drawings: A. Basidiospores; B. Basidia; C. Pleurocystidia; D. Cheilocystidia; E. Caulocystidia	100
Figure 4.3	<i>Suillus indicus</i> (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.	103
Figure 4.4	<i>Suillus indicus</i> , microscopic line drawings: A. Basidiospores; B. Basidia; C. Pleurocystidia; D. Cheilocystidia.	104
Figure 4.5	<i>Suillus himalayensis</i> 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	106
Figure 4.6	<i>Suillus himalayensis</i> , microscopic line drawings: A. Basidia; B. Basidiospores; C. Pleurocystidia; D. Cheilocystidia; E. Caulocystidia	107
Figure 4.7	<i>Suillus granulatus</i> (basidiocarps): A. Scattered growth pattern of	109

	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	
Figure 4.8	<i>Suillus granulatus</i> , microscopic line drawings: A. Basidiospores; B. Basidia; C. Pleurocystidia; D. Cheilocystidia; E. Caulocystidia	111
Figure 4.9a	<i>Suillus sibiricus</i> (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	113
Figure 4.9b	<i>Suillus sibiricus</i> (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	114
Figure 4.10	<i>Suillus sibiricus</i> , microscopic line drawings: A. Basidiospores; B. Basidia; C. Pleurocystidia; D. Cheilocystidia; E. Caulocystidia	116
Figure 4.11	<i>Suillus flavidus</i> (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	118
Figure 4.12	<i>Suillus flavidus</i> , microscopic line drawings: A. Basidiospores; B. Basidia; C. Pleurocystidia; D. Cheilocystidia; E. Caulocystidia	120

Figure 4.13	<i>Suillus placidus</i> (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	122
Figure 4.14	<i>Suillus placidus</i> , microscopic line drawings: A. Basidiospores; B. Basidia; C. Pleurocystidia; D. Cheilocystidia; E. Caulocystidia	123
Figure 4.15	ITS-PCR products of <i>Suillus</i> amplified with ITS1 and ITS4 primers	127
Figure 4.16	ITS-RFLP analysis of <i>Suillus</i> isolates digested with three different restriction enzymes (<i>AluI</i> , <i>HaeIII</i> , and <i>MboI</i>)	128
Figure 4.17	Phylogenetic tree inferred from the bayesian analysis of the ITS region of <i>Suillus</i> species with <i>Rhizopogon subcaerulescens</i> as an out-group taxon	133
Figure 4.18	<i>Suillus</i> isolates (SNW01–SNW08) obtained from the sporocarps collected from different conifer forests of northwestern Himalayas	135
Figure 4.19	Radial growth (cm) of <i>Suillus</i> isolates (SNW01–SNW08) on 2% malt extract agar medium as inferred from means of colony diameter	136
Figure 4.20	Biomass yield (mg/ml) of <i>Suillus</i> isolates (SNW01–SNW08) in 2% malt extract broth medium as inferred from means of mycelium dry weight	136
Figure 4.21	Acid phosphatase activity ($\mu\text{M pNPg}^{-1}\text{h}^{-1}$) of different <i>Suillus</i> isolates (SNW01–SNW08)	138
Figure 4.22	Phytase activity ($\mu\text{M Pig}^{-1}\text{h}^{-1}$) of different <i>Suillus</i> isolates (SNW01–SNW08).	139
Figure 4.23	Protease activity (PUEs) of different <i>Suillus</i> isolates (SNW01–SNW08)	140
Figure 4.24	Chitinase activity ($\text{mM g}^{-1}\text{min}^{-1}$) of different <i>Suillus</i> isolates (SNW01–SNW08)	140
Figure 4.25	Micrographs of pine root tip mycorrhizal with <i>Suillus</i> isolate after four months of the mycorrhization showing hartig net, mantle layer and emanating hyphal network	141
Figure 4.26	Effect of inoculation with different <i>Suillus</i> isolates on ectomycorrhizal root colonization of blue pine (<i>Pinus wallichiana</i>)	142

	seedlings	
Figure 4.27	Effects of varying concentrations of malt extract (g/l) on the radial growth of <i>S. indicus</i> SNW02 and <i>S. sibiricus</i> SNW06	147
Figure 4.28	Effects of various carbon sources on the radial growth of <i>S. indicus</i> SNW02 and <i>S. sibiricus</i> SNW06	148
Figure 4.29	Effects of varying concentrations of glucose (g/l) on the radial growth of <i>S. indicus</i> SNW02 and <i>S. sibiricus</i> SNW06	149
Figure 4.30	Effects of various nitrogen sources on the radial growth of <i>S. indicus</i> SNW02 and <i>S. sibiricus</i> SNW06	150
Figure 4.31	Effects of varying concentrations of di-ammonium hydrogen phosphate (g/l) on the radial growth of <i>S. indicus</i> SNW02 and <i>S. sibiricus</i> SNW06	151
Figure 4.32	Effects of different adenosine concentrations (g/l) on the radial growth of <i>S. indicus</i> SNW02 and <i>S. sibiricus</i> SNW06	152
Figure 4.33	Effect of incubation temperature on radial growth of <i>S. indicus</i> SNW02 and <i>S. sibiricus</i> SNW06	153
Figure 4.34	Effect of pH of medium on radial growth of <i>S. indicus</i> SNW02 and <i>S. sibiricus</i> SNW06	154
Figure 4.35	A. A heap of <i>Pinus wallichiana</i> needles collected from the blue pine (<i>Pinus wallichiana</i>) forest B. Biochar obtained by pyrolysis of <i>Pinus wallichiana</i> needles	156
Figure 4.36	A view of experimental site showing <i>Pinus wallichiana</i> seedlings growing in polypropylene bags	157
Figure 4.37	Root systems of blue pine (<i>Pinus wallichiana</i>) seedlings at the end of the nursery stage: A. Roots colonized with <i>S. indicus</i> SNW02 B. Roots colonized with <i>S. sibiricus</i> SNW06	157
Figure 4.38	<i>Suillus indicus</i> ectomycorrhizae on <i>Pinus wallichiana</i>	158
Figure 4.39	<i>Suillus sibiricus</i> ectomycorrhizae on <i>Pinus wallichiana</i>	160

List of Tables

Table 4.1	Different <i>Suillus</i> specimens collected from three different states of north western Himalayas, India along with collection site, date of collection and forest type	96
Table 4.2	Different <i>Suillus</i> cultures isolated from the northwestern Himalayas, India	126
Table 4.3	Differentiation of <i>Suillus</i> isolates into RFLP types according to the ITS-RFLP patterns produced following digestion of the ITS-PCR product with restriction enzymes.	129
Table 4.4	Examined <i>Suillus</i> species and their closest relative species inferred from ITS gene sequences of existing database	130
Table 4.5	Size of restriction fragments produced following digestion of the ITS-PCR products of <i>Suillus</i> isolates with different restriction enzymes. Exact size of fragments was deduced using web programme Web Cutter 2.0	131
Table 4.6	Extracellular enzyme activities of different <i>Suillus</i> isolates primarily involved in phosphorus and nitrogen uptake	137
Table 4.7	Influence of inoculation with different <i>Suillus</i> isolates on growth and biomass of blue pine (<i>Pinus wallichiana</i>) seedlings after four months of the growth period	143
Table 4.8	Influence of inoculation with different <i>Suillus</i> isolates on nutrients content of blue pine (<i>Pinus wallichiana</i>) seedlings after four months of the growth period	145
Table 4.9	Radial growth of <i>S. indicus</i> SNW02 and <i>S. sibiricus</i> SNW06 grown in different media	146
Table 4.10	Radial growth and biomass yield of <i>S. indicus</i> SNW02 and <i>S. sibiricus</i> SNW06 in basal 2% malt extract (ME) medium and respective optimized media	155
Table 4.11	Effects of mycorrhizal inoculants and biochar amendment on mycorrhizal root colonization and plant growth of <i>Pinus</i>	161

	<i>wallichiana</i> seedlings	
Table 4.12	Effects of mycorrhizal inoculants and biochar amendment on nutrients content of <i>Pinus wallichiana</i> seedlings	163
Table 4.13	Effects of mycorrhizal inoculants and biochar amendment on physico-chemical properties of soil	164
Table 4.14	Effects of mycorrhizal inoculants and biochar amendment on soil enzyme activities	166
Table 5.1	Ecology and distribution of seven different <i>Suillus</i> species identified from the northwestern Himalayas	169

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 ₂ O 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	Mitochondrial 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 ₄ OH	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