Saudi Journal of Biological Sciences 27 (2020) 524–534

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Identification and inoculation of fungal strains from *Cedrus deodara* rhizosphere involve in growth and alleviation of high nitrogen stress

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ARTICLE INFO

Article history:
Received 19 September 2019
Revised 13 November 2019
Accepted 17 November 2019
Available online 26 November 2019

Keywords:
Fungal strains
Cedrus deodara
Nitrogen
Expansins
Cystatin
Enzymes

ABSTRACT

Cedrus deodara is economically and ethnobotanically an important forest tree and is shown to be at decline in Northern areas of Pakistan in recent years mainly due to high concentration of Nitrogen in forests. Ectomycorrhizal (ECM) association forming fungi enables the forest trees to develop optimally by absorbing water from the rhizosphere through their absorptive hyphae and by making available the nutrients by mobilization of N and P from the organic substrates. This study was conducted to identify the ECM strains from *C. deodara* rhizosphere and to analyse the impact of high N load on the *C. deodara* seedlings to establish N critical load value for coniferous forests of Pakistan. Six new fungal strains were identified from the rhizosphere of *C. deodara* and were registered at GenBank (NCBI) as *Emmia latemarginata* strain ACE1, *Aspergillus terreus* strain ACE2, *Purpureocillium lilacinum* strain ACE3, *Talaromyces pinophilus* strain ACE4, *A. fumigatus* strain ACE5 and *T. pinophilus* strain ACE6 with accession numbers MH145426, MH145427, MH145428, MH145429, MH145430 and MH547115. Four out of six isolated strains were inoculated with seedlings of *C. deodara* singly and in consortium (CN) in combination with nitrogen load of 0 (C), 25 (T1), 50 (T2), 100 kg N ha^{-1} yr^{-1} (T3). Agronomic, physiological and gene expression studies for *ExpansinA4* (*EXPA4*) and *Cystatins* (*Cys*) were made to analyse the impact of fungal strains in relation to high N stress. This study suggests a positive impact of T1 (25 kg N ha^{-1} yr^{-1}) Nitrogen load and a negative impact of T3 (100 kg N ha^{-1} yr^{-1}) on growth parameters and expression patterns of *EXPA4* and *Cys* genes. Peroxidase (POX) activity decreased in the order ACE5 > ACE2 > C > ACE3 > ACE1 > CN. However, the results of Superoxide dismutase (SOD) showed decreasing trend in the order ACE5 > C > CN > ACE1 > ACE2 > ACE3. Strain ACE3 was shown to have a positive impact on the seedlings in terms of growth, physiology and expression of genes. Present study suggests that newly identified fungal strains showing positive impact on the growth and physiology of *C. deodara* could be used for the propagation of this economically important plant in Pakistan after pathogenicity test.

1. Introduction

Mycorrhizae is the evolved symbiotic association between fungi and the roots of higher plants where the associated fungi assist the plants to uptake nutrients and water from the soil, and in return take carbon (C) as a source of food. Mycorrhizae is responsible for water and nutrient uptake for 86% of the plant species (except for plants belonging to *Brassicaceae, Cyperaceae, Caryophyllaceae, Juncaceae*; being non-mycorrhizal) (Rillig et al., 2015). Mycorrhizae association is of different types including extracellular, intracellular and ecto-endo mycorrhizae also known as ectomycorrhiza,

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2019.11.016
1319-562X/

ericoid mycorrhizae and arbutoid mycorrhizae. Ectomycorrhiza (ECM) accounts for about 95% of forest plant species, formed primarily by the members of Basidiomycota, Ascomycota and Zygomycota (Rinaldi et al., 2008, Becquer et al., 2014). A huge number of plants and fungal species are involved in ECM and their number is increasing day by day with the new species being identified and old species being excluded by further experimentation proving their non-ECM status (Fukasawa et al., 2019). The ECM fungi forms a soil-fungus-root interface in the rhizosphere around short lateral roots of the plant and a network of hyphae termed as extra-radical mycelium, helps in translocation of water and minerals specifically Nitrogen (N), Phosphorus (P) and Potassium (K) by mobilizing them from the organic substrates to the host plant in exchange for carbohydrates as a C source to gain energy (Clasen et al., 2018). ECM fungi release the extracellular enzymes that degrade the complex organic matter from the soil like proteins, cellulose, hemicellulose, lignin, and chitin thus releasing N, P that are taken up by the plants. ECM fungi also enhance the photosynthetic processes of the host plants by increasing the carbon sink strength of ECM roots and directing the photo-assimilates toward the host roots (Rathnayake et al., 2007). It has been hypothesized that the decline in N, photo-assimilates (carbohydrates) and other nutrient (P, K) in the decomposing organic matter containing soil may have exerted a pressure on the saprophytic fungi to form symbiosis with the roots of forest plants to acquire the food in return for water and nutrients (Martin et al., 2016). ECM forms association with the lateral roots of plants by supressing the host defence response after releasing the effector protein known as Mycorrhizal induced small secreted protein 7 (*MiSSP7*). It is secreted extracellularly by the hyphae after receiving the plants signal and is the first gene known to be essential for ECM association. Interacting with the transcriptional repressor gene JAZ6 (Jasmonate Zim Domain protein 6), *MiSSP7* limits the jasmonate induce defence response in host plant by inhibiting the jasmonate-inducible genes and facilitating root colonization (Martin et al., 2016).

The cedar specie *Cedrus deodara* also known as the deodar cedar or Himalayan cedar is a large evergreen coniferous plant native to the Western Himalayas in Northern Pakistan, Eastern Afghanistan, North-Central India, South Western Tibet and Western Nepal lying at altitudes between 1500 and 3200 m and reaching 60 m in height (Ahmed et al., 2011). The deodar is the national tree of Pakistan and is generally cultivated in the areas with mild winters, whereas these trees cannot survive the temperatures below −25 °C. The most cold-tolerant species is found in the Northwest of Kashmir and Pakistan. *C. deodara* is known to be a host to many ECM fungi and has been shown to form ECM association specifically with *Boletus edulis, B. hoarkii* and *Octaviania densa* (Lakhanpal, 2000). Hanif et al. (2012) reported for the first time, five new ECM species associated with deodar namely, *Russula livescens, Peziza* sp. MHSUC-01, *Tomentella* sp. 2ENA19_11, *Tomentella* sp. ENA35_13 and *Tomentella* sp. 2ENA35_13.

Nitrogen is the most abundant element in the atmosphere with a percentage of ~78%. N is a limiting nutrient for the growth, development and metabolism of the plants being a component of chlorophyll; needed for photosynthesis, proteins; component of amino acids, enzymes, DNA; as vital part of nitrogenous bases, adenosine triphosphate (ATP) for energy storage and transfer (Bobblink et al., 2003). Many studies have reported that nitrogen availability largely impacts bacterial and fungal communities (Guo et al., 2019). It has been hypothesized that the atmospheric deposition of N has increased since the Industrial Revolution of second half of 20th century (Zhang et al., 2019). Atmospheric deposition of N has resulted from the emission of ammonia (NH_3) and Nitrogen oxides (NOx) from intensive agriculture practices and fossil fuel consumption respectively to sustain the increasing demands of population increase (Galloway, 2001). Current NOx emission rates are measured to be 8 times the natural emission rate and are estimated to reach 200 Tg N yr^{-1} by the year 2050 (Bytnerowicz et al., 2013; Prospero et al., 1996). The N critical load for Europe has been set to be 15–20 kg N ha^{-1} yr^{-1} for coniferous and deciduous species (Bobblink et al., 2003). Average rate of wet and dry deposition of N from the atmosphere are estimated to be 22 kg N ha^{-1} yr^{-1} exceeding a maximum value of 50 kg N ha^{-1} yr^{-1} for East Asia, especially Japan points toward a possibility of adverse impact of N deposition on Asian forests (Yamaguchi et al., 2007). According to Izuta and Nakaji, (2003), global estimates of the emissions of NOx and NHy are approximately 52 and 109–131 Tg N yea r^{-1} respectively. Excessive deposition of N from the atmosphere acts as phytotoxicant causing soil acidification (due to elevated nitrification with high NH_4^+input to the soil) and eutrophication in forest ecosystems, disintegrating nutrient status such as excessive accumulation of Manganese (Mn) or deficiency of Phosphate (PO_4^{3-}) and Magnesium (Mg) in leaves, and increased sensitivity to other environmental stresses such as drought, frost and gaseous air pollutants (Izuta et al., 2005; Mackay et al., 2017; Yamaguchi et al., 2007). During the soil acidification process, soils release base cations of Calcium (Ca^{2+}) and Mg^{+2}, neutralizing the increase in acidity. Once these base cations have been depleted, Aluminium (Al) is released from the soils, often reaching toxic levels. High accumulation of NH_y due to agricultural activities results in uncoupling of electron transport, membrane dysfunction and appearance of visible plant injury (Izuta and Nakaji, 2003; Cooke & Weih, 2005). Consequently, plant suffer from nutrient deficiency, cationic imbalance and excessive accumulation of Mn and Al, damaging roots of plants; reducing the nutrient uptake leading to detrimental effect on plant.

In Pakistan, the molecular identification and physiological study of symbiotic association of *C. deodara* and its associated ECM has not been undertaken, neither did the impact of high N evaluated as a possible cause of forest decline. Therefore, limited information is available in these regards. Current study has focused on identifying strains of ECM and its association in the rhizosphere of *C. deodara* and effect of high N load on its association at molecular level.

2. Materials and methods

2.1. Sample collection

Two-year-old seedlings of *C. deodara* were acquired from Billion Tree Tsunami Afforestation Project Nursery (BTTAP) Salhad, Abbottabad. These seedlings were maintained in Biotic & Abiotic Stress, transcriptomic & Proteomics Lab for 1 month prior to experimentation under 14L: 10D photoperiod (300 μmol photons m^{-2} s^{-1} equivalent to 15.12 mol photons m^{-2} d^{-1}) at 25 °C (Nara, 2006). Seedlings were watered regularly with tap water and soil samples were collected from the rhizosphere of *C. deodara* from Degchay ka Kattha, Kund, Gatti ka Nakka in Thandiani forest Abbottabad, Pakistan. Soil samples were collected at a depth of 15–20 cm in zipper storage bags and a composite of these samples were made for analysis of fungal isolation soil sample, soil analysis and nitrogen stress sample for planting seedlings.

2.2. Soil analysis

Soil analysis was done to measure the pH and concentration of nitrates and heavy metals in the soil. pH of each soil sample was measured in situ by adding 10 g of soil to 25 ml of water, the mixture formed was homogenized for 1 h. pH was measured using portable pH meter. The pH meter was calibrated using acidic (4), neutral (7) and basic (10) solutions for exact readings. Three readings were taken and a mean of the three was recorded in the log

book (Asemoloye et al., 2017a). Heavy metal analysis was done for the concentration of Zn, Cu, Ni, Co, Cr and Fe in the soil sample using acid digestion method. 3 g of soil sample was digested in a crucible with 10 ml of nitric acid (HNO_3). Mixture was oxidized by heating on hot plate for 1 h. Sample was then allowed to cool at room temperature. After proper cooling, 5 ml of $HClO_4$ was added to the sample. Sample was reheated until a clear digest appeared. Samples were passed through 0.22 mm filter paper. Filtrate was transferred to a volumetric flask and washed with 50 ml of deionized distilled water. Supernatant was made to a total volume of 50 ml and then used for the analysis of mentioned heavy metals through atomic absorption spectrometry (Rashid et al., 2016).

To calculate mineral N (NO_3) content, soil sample was shade dried in the lab. 2.5 g of the dried sample was mixed with 25 ml of distilled water and the obtained mixture was shaken at 175 rpm at 25 °C for 1 h. Mixture was filtered and filtrate was then used for soil nitrate content analysis. 250 μl of 1 N HCl was added to 11.5 ml of filtrate and a homogenous mixture was formed that was analysed at wavelength of 220 nm in spectrophotometer against the standards.

2.3. Isolation and identification of fungal strains

The isolation of fungi from soil was done by serial dilution method. Soil serial dilutions of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were prepared and 20 μl, 30 μl and 50 μl dilutions were spread on potato dextrose agar (PDA) which was prepared by adding 39.1 g in 1000 ml of distilled water at pH of 5.3–5.6 and then sterilized in autoclave. Media was supplanted with 1% (25 μg/ml) of streptomycin sulphate solution to restrict bacterial interference and plates were incubated at 28 ± 7 °C for 7 days. Pure cultures were obtained by sub-culturing using PDA as described above. Morphological and microscopic identification were done using parameters such as colony form, color, elevation, margin and reverse color of the colony on PDA media and hyphae, conidia, conidiophores, spores and hyphal aggregates were also recorded for each strain.

Molecular identification of fungal strains was done through the amplification of NS1 and NS8 regions of fungal rDNA. Fungal DNA was extracted using cetyltrimethylammonium bromide (CTAB) method. The isolated DNA was PCR amplified using universal fungal primer pairs of NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS8 (5′-TCCGCAGGTTCACCTACGGA-3′). Amplified products were sent to Korea (*Macrogen Korea 10F, 254 Beotkkot-ro Geumcheon-gu, Seoul 08511, Rep. of Korea*) for sequencing. The obtained sequencing results for each strain sequence were compared with available sequences at National Centre of Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) program (Asemoloye et al., 2017b). All the newly strains identified were registered on GenBank (NCBI). Subsequently, each newly identified strain was compared with most similar sequences and their phylogenetic relationship was studied by constructing phylogenetic dendrogram using MABL (Phylogeny.fr) tool.

2.4. Fungal inoculation and high nitrogen stress

For fungal inoculation soil samples were autoclaved to avoid any contamination. Tryptic soy broth (TSB) was prepared for the inoculation of seedlings with the isolated strains for the evaluation of the impact of fungal strains on growth and physiology of seedlings. Roots of *C. deodara* seedlings were sterilized with 3% H_2O_2 for 30 min for complete sterilization. After sterilization the seedlings were dipped in broth media and then planted in autoclave soil in pots. The planted seedlings were maintained in the lab under 14L: 10D photoperiod (300 μmol photons m^{-2} s^{-1} equivalent to 15.12 mol photons m^{-2} d^{-1}) at 25 °C. Seedlings were harvested after 30 days for the determination of expression of genes and antioxidant enzymes. N stress in the form of ammonium Nitrate (NH_4NO_3) was given to the seedlings. Four high N stress treatments were applied for each of the experimental study as 0 kg N ha^{-1} (C), 25 kg N ha^{-1} (T1), 50 kg N ha^{-1} (T2) and 100 kg N ha^{-1} (T3), equivalent to 0 mg l^{-1}, 36.25 mg l^{-1}, 72.5 mg l^{-1} and 145 mg l^{-1} fresh soil volume respectively. All the experimentation was done in three replicates in separate pots. Seedlings were watered with deionized water as necessary.

2.5. Stem height and diameter measurement

Plant height and diameter was measured before and after fungal treatment and stress treatment as well. Stem height and diameter of six fungal treatments i.e. Control (C) without inoculation of fungal strain, five treatments of ACE1, ACE2, ACE3, ACE5, CN (consortium) strains and four stress treatments, C (control), T1, T2, T3 were measured. Height was measured in centimetres (cm) from the cotyledon scar on the seedling to the tip of the shoot using measuring tape. Diameter was measured from the cotyledon scar using a micrometre screw gauge (zero error of −0.1 mm corrected) in millimetres (mm).

2.6. Nucleic acid extraction

Total RNA extraction was carried out by CTAB method followed by (Yee et al., 2018) with some modifications. Frozen plant material was grounded in pre-warmed CTAB buffer and incubated at 65 °C for 15 min. Subsequently samples were spun at 12000rcf for 15 min. Supernatant was separated out in new Eppendorf, and again spun at same conditions after addition of chloroform: isoamyl alcohol (24:1). Supernatant was taken out into new Eppendorf and this step was repeated 2–3 times to remove all impurities. Finally, 10 M LiCl was added and incubated overnight at −20°. Next day samples were again spun at same conditions. Pellet formed was washed with 70% ethanol and suspended in RNase free water. RNA concentration and quality were determined using spectrophotometer and by agarose gel electrophoresis.

2.7. Reverse transcriptase-Polymerase chain reaction (RT-PCR)

Degenerate primers were designed for three genes i.e. ExpansinA4 (EXPA4) and Cystatin (Cys), because of the unavailability of sequenced genome of *C. deodara* (Table 1). Expression of these genes was analysed in needles of all experimental plants by using PCR program. Expression of these genes were then compared with housekeeping gene (RS-Actin). cDNA was prepared from 5 μg of RNA using oligo (dT) primer and reverse transcriptase enzyme (Enzynomics, SeoμL). RT reactions were incubated at 42 °C for 5 min and were stopped by heating at 95 °C for 5 min. Fragments of expansins, cysteine protease, cystatin and references genes were PCR-amplified from 50 to 100 ng of cDNA equivalent, using degenerate primers.

2.8. Antioxidant enzyme assay

Antioxidant enzyme assay was done to determine the physiology of the seedlings as with the inoculation of fungal strains. For antioxidant enzyme assay, following buffer solutions were prepared: 0.025 M sodium pyrophosphate buffer by adding 660 mg sodium pyrophosphate in 90 ml of deionized water (pH 8.3) up to final volume of 100 ml, potassium-phosphate buffer was prepared by adding 9.5 g of KH_2PO_4 and 5.25 g of K_2HPO_4 in 90 ml of distilled water (pH 6.5). Solution was made to a final volume of 100 ml, 1X phosphate buffer was prepared by adding 1.44 g of Na_2HPO_4, 8 g NaCl, 0.2 g KCl, and 0.2 g of KH_2PO_4 in 900 ml of dis-

Table 1
Primers used in this study for expression analysis of Expansins and Cystatins genes.

Gene name	Primers	Sequence
ExpansinA4	Forward	5′-AGAATYCCYGGBGTTTAC-3′
	Reverse	5′-CTTGAGGAACATRGGCAT-3′
Cystatin	Forward	5′-GAACAGGTTGTGGCGGGTACA-3′
	Reverse	5′-TAGGGAATTAGATCTTTGCTG-3′
Actin	Forward	5′-GCATCACACTTTCTACAAC-3′
	Reverse	5′-CCTGGATAGCAACATACAT-3′

tilled water that was made to a final volume of 1 litter. 1 M HCl was used to adjust pH to 7.4.

Enzyme extract was prepared by grinding 0.5 g of *C. deodara* needles in 3 ml of 50 mM potassium phosphate buffer. The ground substance was then centrifuged at 2000*g* for 10 min and supernatant was taken into new Eppendorf and was used for the assay. For Superoxide Dismutase (SOD), Assay mixture was prepared by mixing 1.2 ml of 0.025 M sodium pyrophosphate buffer, 0.1 ml of 186 μM phenazine methosulphate, 0.3 ml of 300 μM nitro blue tetrazolium and 0.2 ml of the enzyme extract. Water was added to make volume up to 2.8 ml. The reaction was initiated by addition of 2 ml of 780 μM NADH. Mixture was incubated at 30 °C for 90 min. Enzymatic reaction was arrested by addition of 1 ml of glacial acetic acid and subsequently shaken with 1 ml of n-butanol. Reaction mixture was incubated for 10 min at room temperature and then centrifuged. After incubation, colour was produced in butanol layer whose intensity was determined by spectrophotometer at wavelength of 560 nm. The activity of SOD was measured in units/mg using the formula:

$$\%\ \text{Inhibition} = \left\{\frac{\text{Control OD} - \text{Treatment OD}}{\text{Control OD}}\right\} \times 100 \qquad (1.2)$$

Percent inhibition was then converted to units/mg fresh weight of SOD activity.

For peroxidase (POX), 20% plant extract homogenate was prepared in 0.1 M phosphate buffer (pH 6.5) containing 1% H_2O_2. Homogenate was centrifuged at 2000*g* for 10 min and supernatant was used for assay. For the reaction to carry out, 1 ml of extract was added to the 3 ml of pyrogallol. Absorbance of the product (purpurogallin) formed was measured at 430 nm after adding 0.5 ml H_2O_2 into the mixture. Increase in absorbance was measured after 30 sec till 2 min. Before the assay instrument was adjusted to zero at 430 nm.

One-unit POX activity is defined as, change in absorbance/min at λ = 430 nm

$$\text{units/mg fresh weight} = \frac{500}{\Delta T} \times \frac{1}{1000} \times \frac{\text{total volume}}{\text{volume used}} \times \frac{1}{\text{weight of sample}} \qquad (1.3)$$

3. Results

3.1. Identification of isolated fungal strains

The isolated fungal strains were identified based on morphological, microscopic and molecular characteristics. Morphologically the strains were identified based on the colony's morphology, colour, size, and elevation on PDA media (Fig. 1). The microscopic characteristics that were recorded for each strain included the hyphae type, hyphal aggregates, fruiting bodies and spores shown in (Table 2). The morphological and microscopic attributes were then used for the molecular identification of the strains to the highest percent similar sequence query on NCBI database. The query that gave the highest query cover with highest similarity in terms of sequence and morphology was selected. All the new strains were then registered on GenBank (NCBI) and coded as ACE1, ACE2, ACE3, ACE4, ACE5 and ACE6 (Table 3). These strains were identified as *Emmia latemarginata* strain ACE1 (Accession no. MH145426), *Aspergillus terreus* strain ACE2 (Accession no. MH145427), *Purpureocillium lilacinum* strain ACE3 (Accession no. MH145428), *Talaromyces pinophilus* strain ACE4 (Accession no. MH145429), *A. fumigatus* strain ACE5 (Accession no. MH145430) and *T. pinophilus* strain ACE6 (Accession no. MH547115).

Phylogenetic analysis was performed for the confirmation of the strain by using Phylogeny.fr software. This showed the clear relationship of each strain with the closely related strains in Fig. 2A–F. The resulting tree for ACE1 showed the presence of three clusters, indicating a close relation between strain ACE1 and *Oxyporus latemarginatus* (*E. latemarginata*) (Fig. 2A). For ACE2 the tree showed the presence of many clusters, among which strains ACE2 form a cluster with *A. terreus* showing that both evolved from a common ancestor and are closely related to each other (Fig. 2B). Cladogram for ACE3 showed the formation of four clusters. First cluster indicates that there is close relationship between strain ACE3 and *P. lilacinum,* (a member of Ophiocordycipitaceae family in Division Ascomycota) having a common ancestor (Fig. 2C). Phylogenetic tree for ACE4 showed the presence of three clusters. It was interpreted that strain ACE4 and *T. pinophilus* have a common ancestor (Fig. 2D). ACE5 is related to *Aphanoascus cinnbarinus,* Eurotiomycetes and *A. fumigatus*. But the strain was identified to be as *A. fumigatus* strain ACE5 because of the morphological similarity on PDA media (Fig. 2E). ACE6 forms a cluster with *T. pinophilus* indicating a close relationship between the two. Therefore, strain ACE6 was identified as *T. pinophilus* strain ACE6 (Fig. 2F).

3.2. Soil analysis

Soil analysis was done to analyze the pH, soil texture and concentration of NO_3, Fe, Zn, Cu, Cr and Ni in the soil. pH of the soil was measured to be 7.48 that led to the interpretation that soil has a neutral pH, being optimum for the growth of *C. deodara.* Texture of the soil was clayey loam with 30–35% clay content with a high organic matter content. Soil was of dark brown colored when moist and had medium plastic consistency. Soil had 195.27 mg/kg of nitrates and the concentration of the heavy metals were in the order of Fe > Cr > Ni > Zn > Cu > Co. Results for soil characterization indicated that concentration of Zn, Cu, Cr and Ni were in the safe limits for optimum growth of the seedlings, but a high value of Fe and Co was observed (Fig. 3).

3.3. Plant height and diameter measurement

Stem height measurement in case of inoculation of fungal strains showed 0.90%, 0.29%, 0.94%, 0.15%, 1.30%, 0.63% increase in C, ACE1, ACE2, ACE3, ACE5 and CN respectively. Data showed highest percent increase in case of ACE5 and lowest in case of ACE3. Results have demonstrated that percent increase in order of ACE5 > ACE2 > C > CN > ACE1 > ACE3. For the estimation of impact of high N stress on the seedlings, the fungal treatments were inoculated with four N stress treatments i.e. 0 kg N ha^{-1} (C), 25 kg N ha^{-1} (T1), 50 kg N ha^{-1} (T2) and 100 kg N ha^{-1} (T3). Stem height for inoculation of strain ACE1, ACE2, ACE3 and ACE5 showed that C treatments had no significant impact on height percent increase, but a high percent increase was observed in stem height in the order of T1 > T2 > T3 for all the single strain inoculation treatments (Fig. 4).

Stem diameter measurement for the impact of fungal strains on growth of *C. deoadara* seedlings showed 1.87% decrease in case of C. Other treatment showed increase in stem diameter i.e. 0.30%,

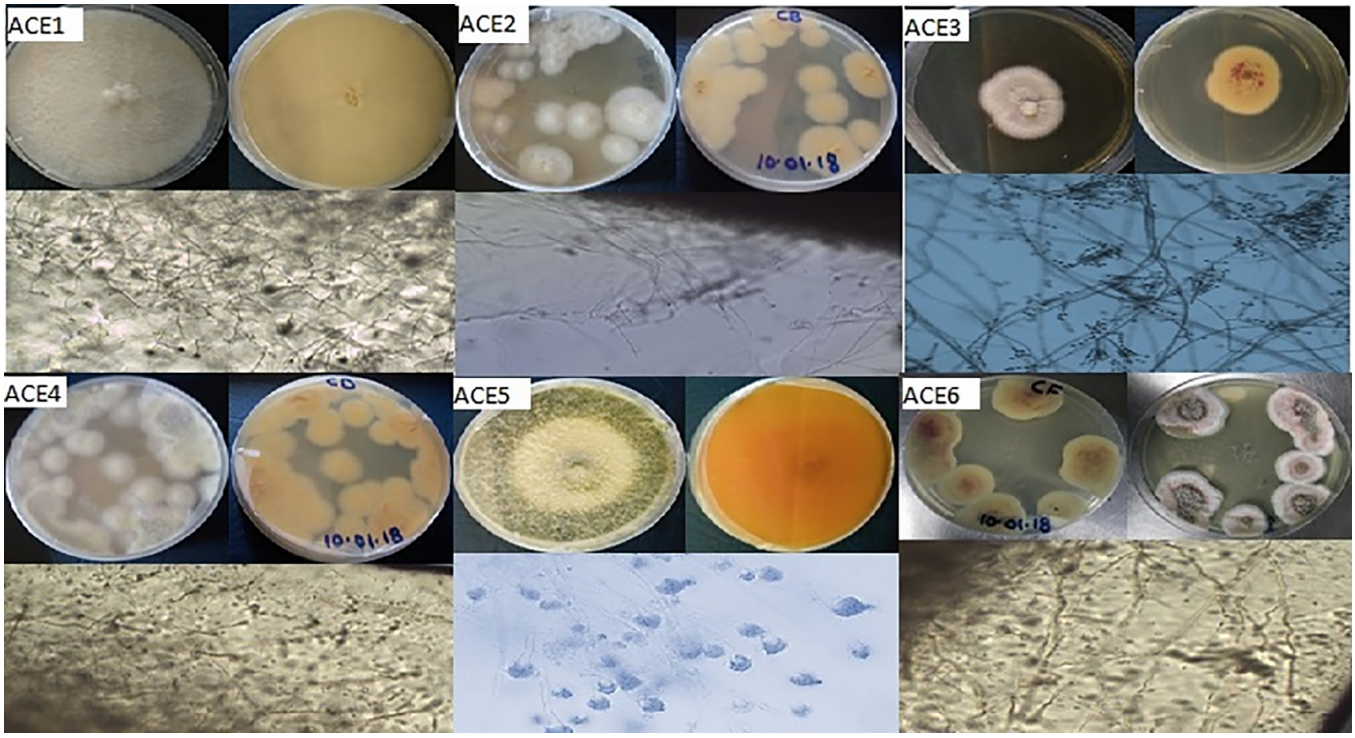

Fig. 1. Morphological characteristic of each isolated fungal strain on PDA media.

Table 2
Morphological parameters of colonies of isolated fungal strains on PDA media.

Isolate	Diameter (mm)	Form	Color	Margin	Elevation	Colony Reverse
ACE1	90	Circular	Whitish	Entire	Raised	Pale
ACE2	35–50	Circular	Edges white, center yellow	Entire	Raised	Pale to light lemon
ACE3	15–50	Irregular	White edges, light pink center	Entire	Raised	Pale to yellow, furrowed
ACE4	20–60	Irregular	Edges white, yellow, center green	Undulate	Umbonate	Furrowed, pale to lemon
ACE5	90	Circular	Greenish, white center	Entire	Flat	Lemon yellow
ACE6	25–35	Irregular	White to light pinkish at edges, center greenish	Entire	Umbonate	Pale with pinkish center

Table 3
Accession numbers of strains registered on GenBank (NCBI).

Code	NCBI submission ID	Accession number	Name	BLAST similarity (%)
ACE1	SUB3843140	MH145426	*E. latemarginata*	98%
ACE2	SUB3861249	MH145427	*A. terreus*	96%
ACE3	SUB3861252	MH145428	*P. lilacinum*	98%
ACE4	SUB3861254	MH145429	*T. pinophilus*	97%
ACE5	SUB3861255	MH145430	*A. fumigatus*	95%
ACE6	SUB4222828	MH547115	*T. pinophilus*	99%

1.30%, 3.50%, 0.23%, 2.06% increase for ACE1, ACE2, ACE3, ACE5 and CN respectively. Results indicate the highest percent increase in case of ACE3 and a decrease in case of C. The N stress treatment also resulted in the same trend as for stem height with an increase in diameter in case of C and subsequent decrease in diameter of the seedlings in the order of C > T1 > T2 > T3 for strain ACE1, ACE2, ACE3 and ACE5 (Fig. 5).

3.4. Antioxidant enzyme assay

The activity of POX and SOD was measured in the plant samples after their inoculation with fungal strains to establish relationship between the *C. deodara* and the selected fungal strains. POX assay was performed to analyze the concentration of peroxidase in units/mg fresh weight of plant material in the inoculation treatment to establish relationship between *C. deodara* and the selected fungal strains. Assay results indicated a highest value of 2.13 units/mg fresh weight in plants inoculated with strain ACE5, while a lowest value of 0.97 units/mg fresh weight in treatment where consortium was applied (Fig. 6). The peroxidase assay resulted the activity of enzyme peroxidase in the order of ACE5 > ACE2 > C > ACE3 > ACE1 > CN.

To analyse the relationship between *C. deodara* and isolated fungal strains, SOD assay was performed. The results showed highest activity of enzyme SOD in ACE5 with a value of 0.017 units/mg fresh weight with a decreasing trend in the order of ACE5 > C > CN > ACE1 > ACE2 > ACE3. The lowest value in case of ACE3 indicated a positive relationship between *C. deodara* and the *Purpureocillium lilacinum* strain ACE3 as shown in Fig. 6.

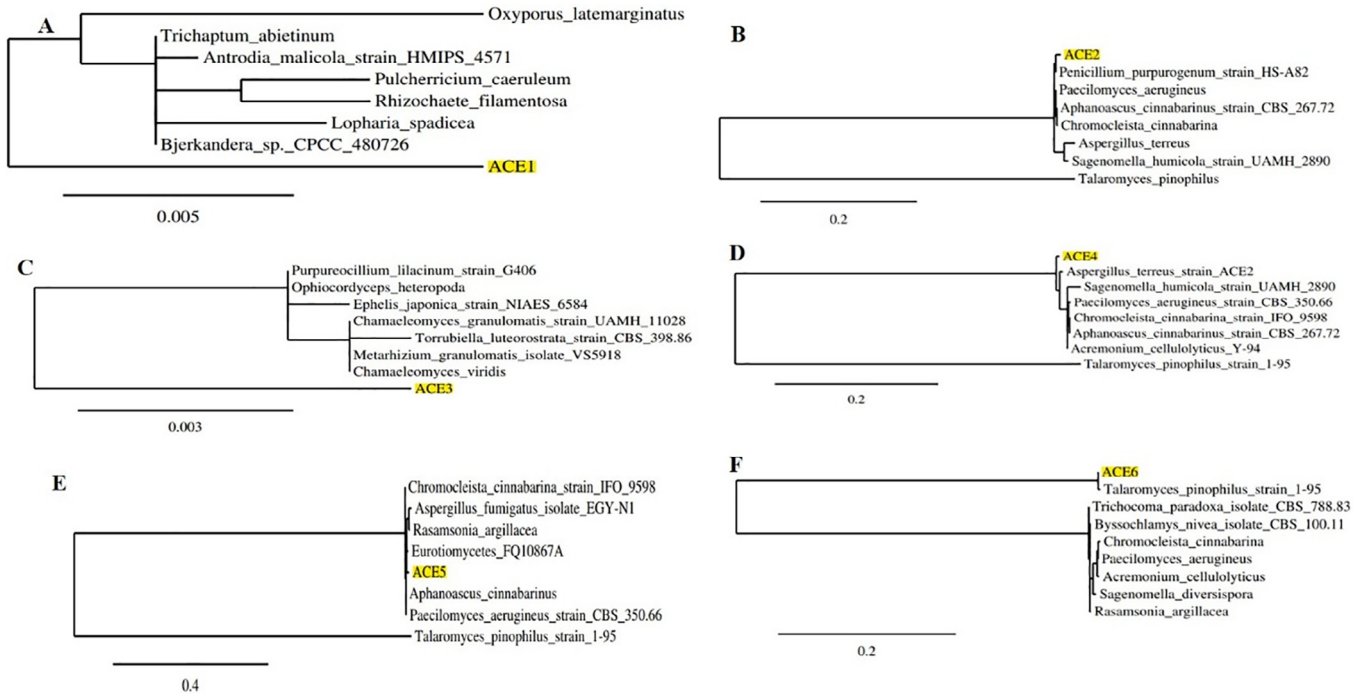

Fig. 2. Phylogenetic analysis of each isolated strain with closely related strain by using Phylogeny.fr software.

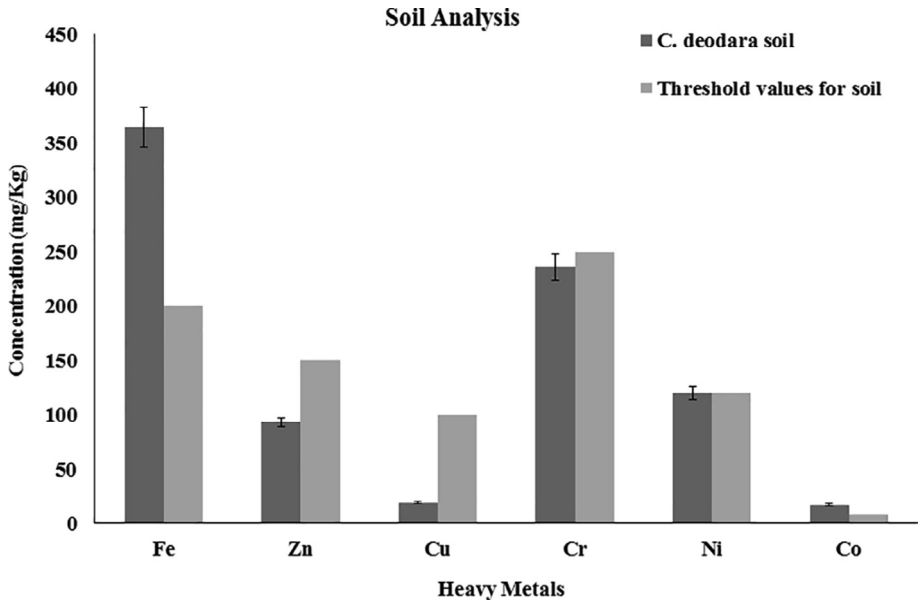

Fig. 3. Heavy metals concentration in the soil of *C. deodara* with threshold level.

3.5. Gene expression analysis

Expression analysis of stress related genes i.e. *Expansins* (EXPA4), *Cystatins* (Cys) was performed for all the treatments including the fungal inoculation experiments and the N stress treatments for all the selected strains.

3.5.1. Expression analysis of Expansins (EXPA4)

Expression study of inoculated fungal strains was done after 30 (1st harvest) and 60 (2nd harvest) days of application of high N stress (Fig. 7A–D). Expression analysis results of EXPA4 in plants inoculated with ACE1 fungal strain after 1st harvest showed that the expression was low in T2 while highest expression was observed in T1. After 2nd harvest the expression was high in all treatments with highest in T3 and least in control. Expression in ACE2 inoculated strains showed increased expression in 2nd harvest as compared to 1st harvest. Decrease expression was observed from C to T3 in 1st harvest but a high expression was seen in T1 and lowest in T2 after 2nd harvest. But overall expression was increased with high N stress (Fig. 7B). Increased expression of EXPA4 for ACE3 inoculated seedlings was observed in all treatments in 2nd harvest. While in 1st harvest the expression of T2 was lowest as compared to control. In 2nd harvest the expression was in order of T2 > C > T1 > T3, which indicates decreased

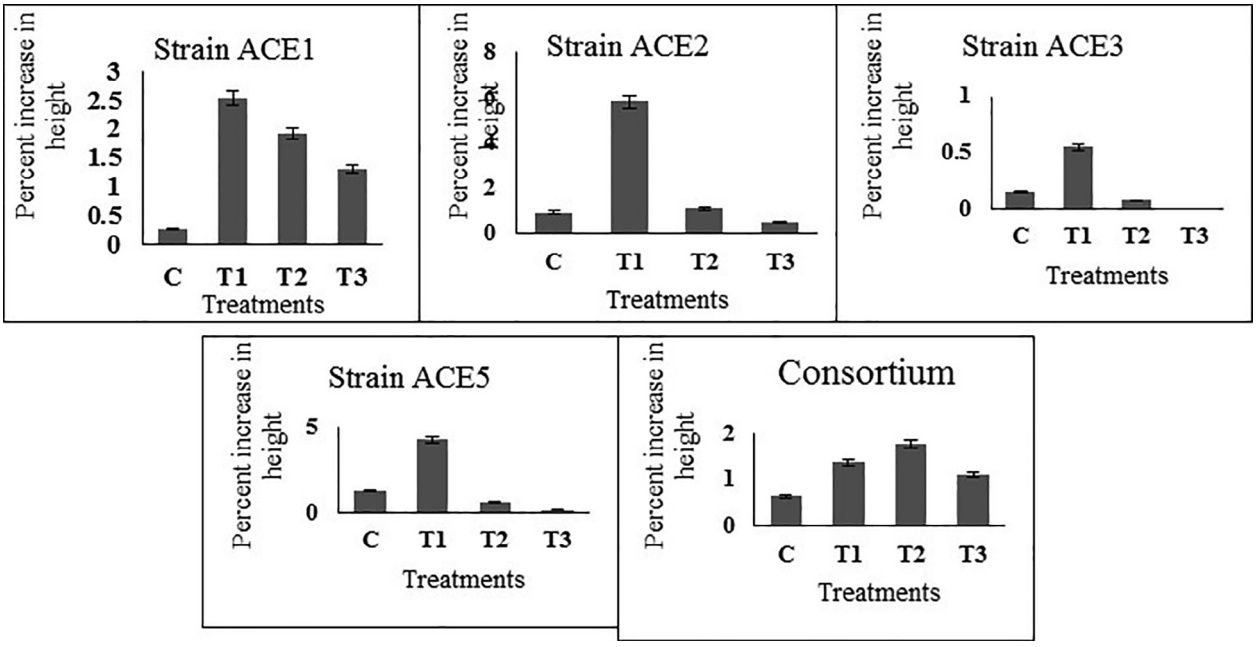

Fig. 4. Percent increase in height in *C. deodara* seedlings inoculated with different fungal strains. Figure showing a decreasing trend in percent height with high Nitrogen stress C = control (0 kg N ha^{-1} yr^{-1}), T1 = 25 kg N ha^{-1} yr^{-1}, T2 = 50 kg N ha^{-1} yr^{-1}, T3 = 100 kg N ha^{-1} yr^{-1}.

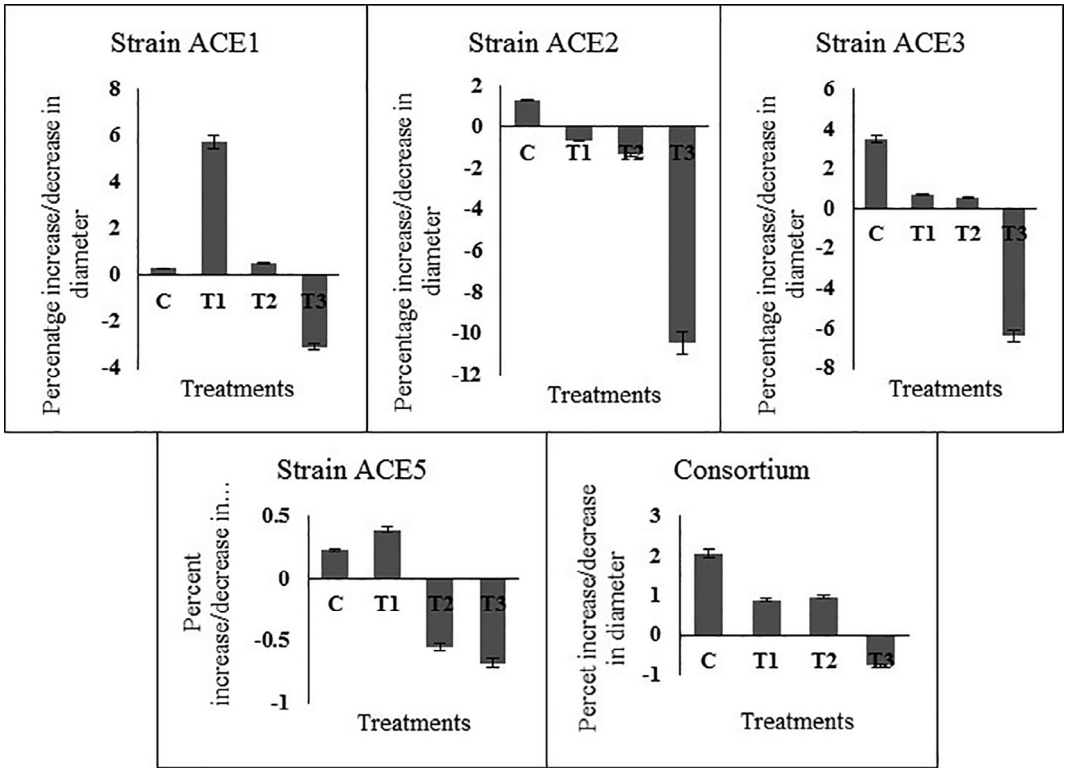

Fig. 5. Percent increase/decrease in diameter in different treatments of fungal inoculation and high Nitrogen stress showing a decrease in percent height for T3 treatment C = control (0 kg N ha^{-1} yr^{-1}), T1 = 25 kg N ha^{-1} yr^{-1}, T2 = 50 kg N ha^{-1} yr^{-1}, T3 = 100 kg N ha^{-1} yr^{-1}.

expression while increased expression in T2 showed optimum growth at 50 kg N ha^{-1} (Fig. 7C). Expression results of EXPA4 for ACE5 inoculated *C. deodara* seedlings showed a marked increase in expression in order of C > T1 > T2 > T3 in 1st harvest indicating a positive relation between ACE5 inoculation along with high N stress. The expression further increased in 2nd harvest but showed fluctuation with high expression in C and a decrease in T1, T2 and T3 (Fig. 7D).

3.5.2. Expression of cystatin

Expression of cystatin was also observed in all fungal inoculated plants. 1st harvest of plants inoculated with ACE1 strain showed no expression in control while low expression was observed in T3. Highest expression was shown by T2 while T1 also showed higher expression as compared to T3. After 2nd harvest, no expression was observed in seedlings (Fig. 7F) while in ACE2 inoculated seedlings the expression was high in T1 and T2 seedlings after 1st har-

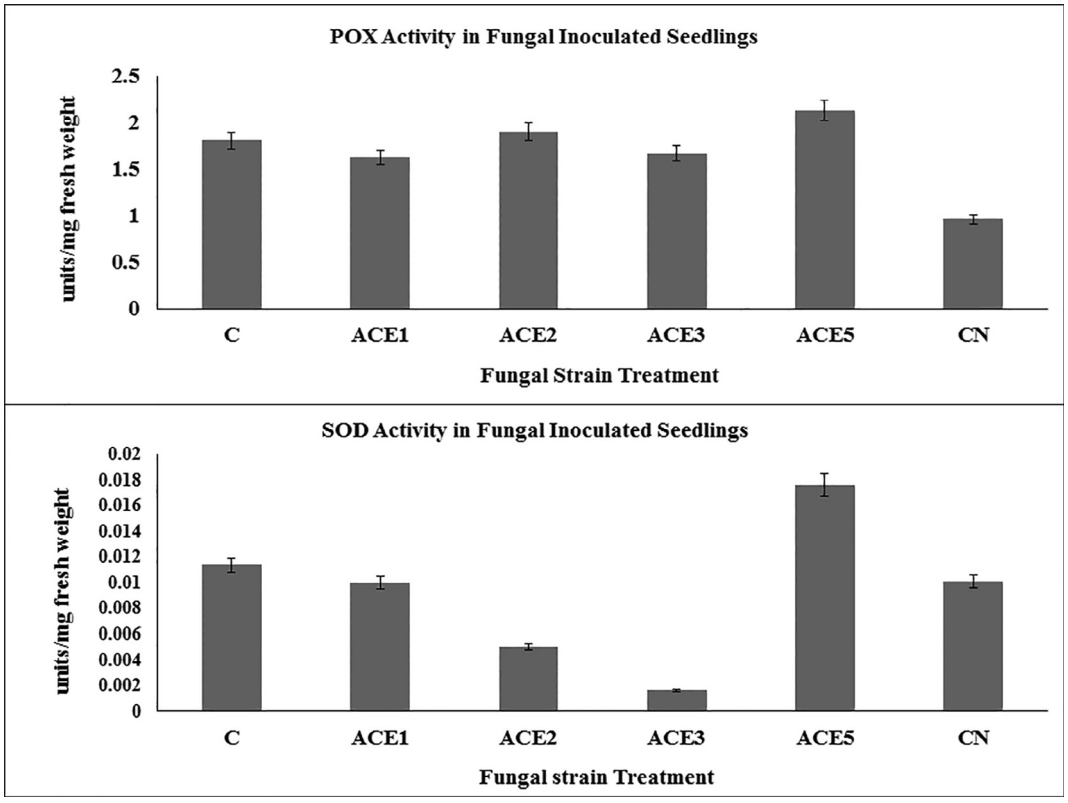

Fig. 6. Peroxidase (POX) and superoxidase (SOD) enzyme activities in *C. deodara* seedlings inoculated with different fungal strains.

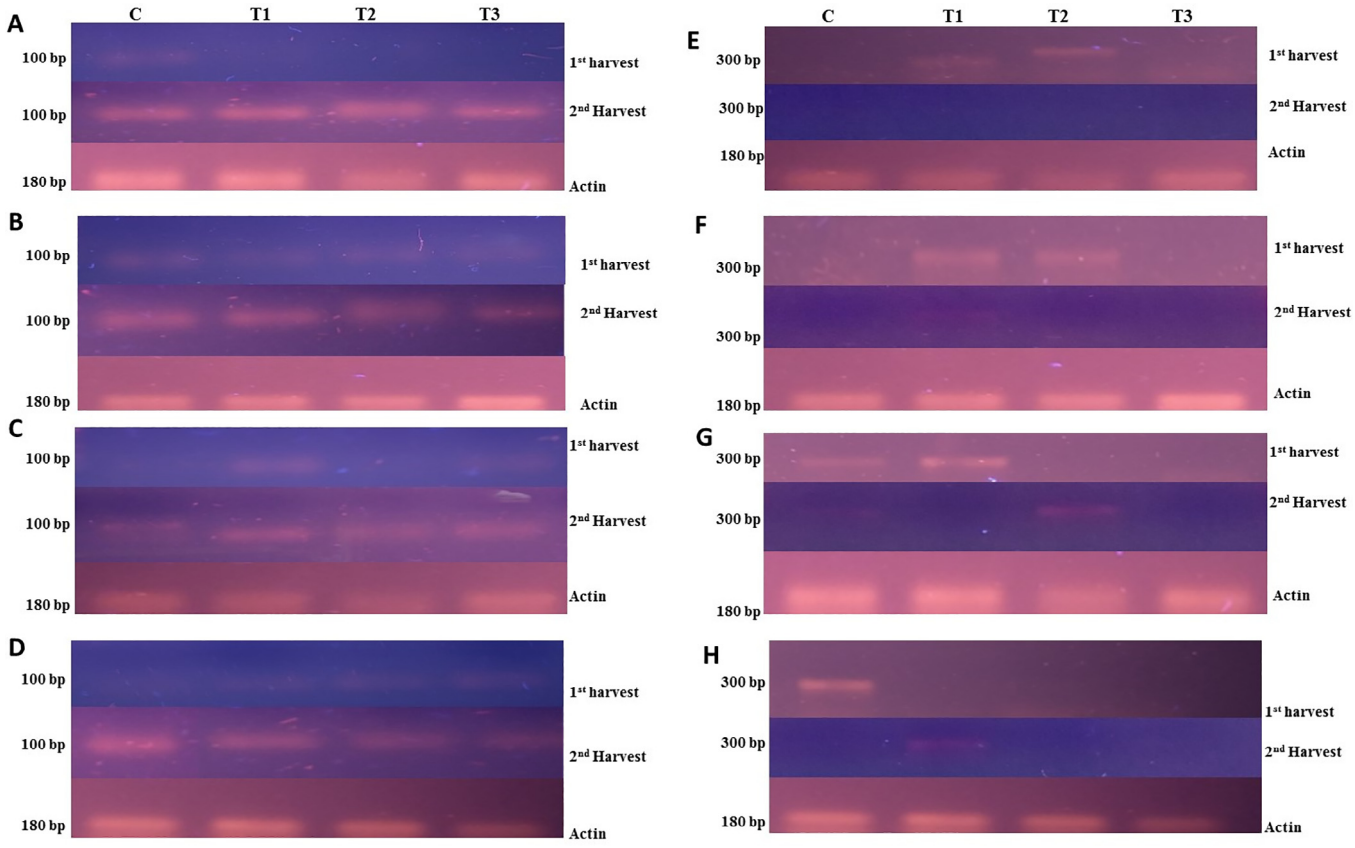

Fig. 7. Expression analysis of expansion (EXPA4) in 1st and 2nd harvest of leaves in comparison with actin gene (reference gene) inoculated with ACE1 (A), ACE2 (B), ACE3 (C) and ACE4 (D) and expression analysis of cystatin (Cys) in 1st and 2nd inoculated with ACE1 (E), ACE2 (F), ACE3 (G) and ACE4 (H) strains.

vest. The bands were not clearly visible for C and T3 on the gel, resulting a marked but decreased expression. After 2nd harvest, the highest but decreased expression as compared to 1st harvest was observed in T1. The expression in case of T2 decreased to almost not visible on the gel (Fig. 7F). Expression analysis of cystatin in ACE3 inoculated seedlings demonstrated an increased expression in T1 in 1st harvest while a decreased expression in C was observed (Fig. 7G). No expression was observed in T2 and T3 on the gel. Expression analysis after 2nd harvest revealed an overall decrease in expression of Cystatin in C and T1. But an increase from almost negligible to high expression was observed in T2. The expression of Cystatin was observed to be highest in C in ACE5 inoculated seedlings after first 30 days of application of high N stress. Low expression in T1, T2 and T3 for cystatin on the gel was observed (Fig. 7H). After 2nd harvest, an increase in expression of Cystatin was visualized on the gel. There was seen a decrease in expression of Cystatin in C, expression increased in T1 and no expression was observed in T2 and T3 for 2nd harvest.

4. Discussion

Nitrogen although a vital component of many biomolecules; is required by the plants in low quantities to be optimally beneficial for their growth and metabolism but if in excess, is detrimental to the plants (Nakaji et al., 2001; Bloom, 2015). Plants inhabiting many natural and semi-natural ecosystems are prone to nutrient poor conditions of low N availability (Bobblink et al., 2003). After the Industrial revolution there has been an increase in anthropogenic emission of N in the atmosphere in the form of NO_x and NH_y resulting in forest decline worldwide (Nakaji et al., 2001). A critical load value of 15–20 kg ha^{-1} yr^{-1} of N has been set for Europe and a value of 50 kg ha^{-1} yr^{-1} of N for East Asian countries have been proposed (Watanabe et al., 2012). Excessive N deposition leads to soil acidification, resulting in reduced availability of essential mineral elements, thus decreasing the plant vitality and forest decline (Izuta et al., 2005). Pakistan although bestowed with world's most diverse ecosystem is short of forest cover area from the International standard of 25%. The study was conducted with the aim to analyse the impact of high N load on the coniferous forest species of Pakistan in terms of growth, physiology and molecular expression of growth-related genes.

About 95% of the forest species rely on the ECM association for their optimum growth (Hanif et al., 2012). Lack of ECM association have been speculated to be a reason for plantation failure for afforestation purposes (Van Der Heijden et al., 2015). Many researchers have reported that pre-inoculation of seedlings with suitable ECM fungi results in better growth of the seedlings, providing a convincing evidence for the crucial role that ECM fungal partner play for the restoration and rehabilitation of degraded forest ecosystems (Itoo and Reshi, 2014). The present study aimed to isolate and identify the ECM partners of *C. deodara* from Thandiani forest; a conserved and less studied area. A total of six fungal strains were isolated from the rhizosphere of *C. deodara* from Thandiani forest. All the isolated strains were identified to be new strains based on their sequence similarity percentage with the already present strains. The strains were registered on GenBank (NCBI) as *E. latemarginata* strain ACE1, *A. terreus* strain ACE2, *P. lilacinum* strain ACE3, *T. pinophilus* strain ACE4, *A. fumigatus* strain ACE5 and *T. pinophilus* strain ACE6 with accession numbers MH145426, MH145427, MH145428, MH145429, MH145430 and MH547115. Ectomycorrhiza of the isolated strains have not been yet described comprehensively for their association with any of the plant species, neither their use as inoculation fungi is known. Four strains out of six isolated strains were selected based on their luxuriant growth on PDA media, for analysis of their impact on the *C. deodara* seedlings alone and in consortium in terms of growth and physiology to establish their ectomycorrhizal potential.

Ectomycorrhiza are known to show negative correlation between root mycorrhization and growth variables suggesting high metabolic costs of mycorrhizal maintenance during the early seedling development (Vonderwell and Enebak, 2000). Stem height measurement showed that a highest percent increase of 1.30% in ACE5 inoculated seedlings and a lowest and lowest 0.15% increase in case of ACE3 inoculation. Stem diameter measurements resulted in highest percent increase of 3.50% in diameter in case of ACE3 and percent decrease of 1.87% in control that contained the seedlings with sterilized roots. These results demonstrate a positive impact of strain ACE5 on height and strain ACE3 on diameter of seedlings and a negative impact of root sterilization on the diameter of seedlings indicating a correlation of roots and fungal strains for absorption of nutrients. The root colonization analysis showed no sufficiently confidence level to designate the inoculated strains to be ECM because of the indistinguishable root characters as only biochemical and molecular techniques can be used to designate the fungal strains to be ECM or non-ECM.

Anthropogenic N deposition can influence the species diversity and capability of fungal species to form ECM association, and sporocarp production as N load of 35 kg N ha^{-1} yr^{-1} reduced sporocarp production in *Russla* spp. and *Cortinarius* spp. (Repáč, 2007). Fungal strains inoculated seedlings were also subjected to high N stress of 0 kg N ha^{-1} (C), 25 kg N ha^{-1} (T1), 50 kg N ha^{-1} (T2) and 100 kg N ha^{-1} (T3). The stem height data for the high N stress treatments showed a small increase in case of C, while a highest percent increase was observed for all the single strain inoculations in T1 treatments ultimately decreasing in the order of T1 > T2 > T3. Same trend was observed for stem diameter in case of inoculation of single strains. In case of fungal consortium, it was observed that the stem height and diameter showed highest percent increase in case of T2 treatment showing a possible antagonistic effect of the inoculated strains at this level of N availability.

Antioxidant enzymes play a pivotal role in plants in scavenging the free radicals produced as a result of biotic and abiotic stresses (Pisoschi and Pop, 2015). The activity of antioxidant enzymes is related to stress and the subsequent production of free radicals (Zandalinas et al., 2018). SODs are the metallo-proteins that catalyse the dismutation of superoxide free radical (O^{2-}) to H_2O_2 and O_2 and are considered as first line of defence against oxidative damage caused by superoxide radical (Elavarthi and Martin, 2010). The activity of SOD was measured in the seedlings inoculated with fungal strains and it was observed in the order of ACE5 > C > CN > ACE1 > ACE2 > ACE3. Ascorbate peroxidase is a H_2O_2 detoxifying enzyme specific to plants and algae that is responsible to protect chloroplasts and other cell organelles from damage caused by H_2O_2 and OH^- radicals (Asada, 2014). POX assay yielded the activity of POX enzyme is the order of ACE5 > ACE2 > C > ACE3 > ACE1 > CN. Antioxidant enzyme assay results demonstrate a positive impact of strain ACE3 and fungal consortium on the physiology of the seedlings as they resulted in decreased activity of SOD and POX respectively, assuming a minimal production of ROS produced as result of stress. While a negative impact of strain ACE5 was shown on *C. deodara* seedlings, resulting in increased production of SOD and POX to scavenge the produced ROS.

Identification and characterization of growth and stress related genes can help to understand the expression pattern of genes in response to stress (Luo et al., 2011). Expansins are the cell wall proteins that mediate the pH-dependent cell wall extension by initiating acid-induced disruption of hydrogen bonds linking cellulose and hemicellulose microfibrils leading to cell growth and stress relaxation (Cosgrove et al., 2002). Expansins are known to induce abiotic stress tolerance in a variety of plant species, but the role that expansins play in tolerance need to be elucidated (Marowa

et al., 2016). Expansins overexpression has been reported to induce stress tolerance in wheat varieties (Zhao et al., 2011), and transgenic tobacco lines overexpressing *TaEXPB23* driven by CaMV promoter (Li et al., 2011), however Kwon et al. (2008) reported that transgenic *Arabidopsis* plants overexpressing *AtEXPB1* and *AtEXP3* became very sensitive to salt stress. Expression of EXPA4 increased in plants in 1st harvest showing a further increase in 2nd Harvest (60 days after the application of high N stress) and a highest increase was shown in T3, T1, T2 and C in case of ACE1, ACE2, ACE3 and ACE5 respectively. Results showed an inconsistent expression of EXPA4 in the seedlings in response to stress and fungal inoculation.

Cysteine protease inhibitors (cystatins) are the proteins that regulate the expression of cysteine proteases in plants. Their expression is shown to cause stress tolerance in plants by inhibiting the proteolysis of proteins by cysteine proteases. Zhang et al. (2008) reported that *A. thaliana* overexpressing *AtCYSa* and *AtCYSb* genes showed enhanced tolerance to drought, salt, oxidative and cold stress. *Jatropha curcas* in response to salt stress showed tolerance when cystatin was overexpressed (Li et al., 2015). The expression of Cystatin was studied in the present study indicating an increased expression of Cystatin during 1st month of application of high N stress that ultimately decreased to almost no expression in some treatments. In strain ACE1, no expression was observed in 2nd harvest indicating a possible activity of cysteine proteases as a result of stress. Strain ACE2 and ACE5 showed stress tolerance till T1 level, while strain ACE3 showed a higher stress tolerance level of 50 kg N ha^{-1} yr^{-1}.

5. Conclusion

The results of the present study suggest a positive impact of high N stress level of T1 (25 kg N ha^{-1} yr^{-1}) and a negative impact of T3 (100 kg N ha^{-1} yr^{-1}) on the growth parameters and expression patterns of *Expansins* and *Cystatins*. The study also provides the expression pattern of growth and stress related genes in relation to the inoculation of *C. deodara* with the isolated fungal strains along with application of high N stress to categorize the strains as beneficial or detrimental for plants. Strain ACE3 is shown to have a positive impact on the seedlings in terms of growth, physiology and expression of growth and stress related genes. Study has shown that increasing deposition of N to the forest ecosystem might be a threat to the growth and physiology of the coniferous forest species such as *C. deodara.* Anthropogenic Nitrogen emission control is recommended for Pakistan to address serious issue of forest decline in Pakistan. Strains identified in the present study should be further analysed for their pathogenicity and facultative association. Moreover, long-term study from seedlings to mature trees needs to be carried out to confirm the ability of strains to form association with *C. deodara.* Furthermore, Antioxidant enzyme assay in response to high N loads only and in combination with fungal strains could also help us to further elucidate the impact of high N stress on *C. deodara* seedlings.

Declaration of Competing Interest

All authors declare that there is no competing and financial interest

Acknowledgements

This study was financially supported by Higher Education Commission (HEC) of Pakistan in National Research Program for Universities (NRPU) projects with project No: 531 1/Federal/NRPU/R&D/HEC/2016. In addition all authors are grateful to the Deanship of Scientific Research at King Saud University for facilitation through project No RG-1440-054.

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