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Role of ectomycorrhizal colonization in enhancement of nutrients for survival of plants collected from mountainous cold stress areas

Aisha Umar[1*], Mai Ali Mwaheb[2], Fuad Ameen[3], Fares Almomani[4], Laurent Dufossé[5] and Marek Gancarz[6,7]

Abstract

Background Ectomycorrhizal (ECM and ECM-like) structures associated with plant root systems are a challenge for scientists. The dispersion pattern of roots within the soil profile and the nutritional conditions are both favourable factors to motivate the plants to make ECM associations.

Results This study discusses the colonization of mycorrhizal associations in *Kobresia* and *Polygonum* species including *Polygonum viviparum*, *Kobresia filicina*, *K. myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens* grown naturally in cold stressed soils of Gilgit-Baltistan (high-altitude alpine Deosai plains), Hazara, Swat, Dir, and Bajaur. Sieved soil batches were exposed to +5 °C (control), -10, -20, -30, -40, -50, -125 °C for 5 h, and selected plants were sown to these soils for 10 weeks under favourable conditions for ECM colonization. Ectomycorrhizal associations were examined in the above mentioned plants. Some ECM fungi have dark mycelia that look like the mantle and Hartig net. Examples of these are *Kobresia filicina*, *K. myosuroides*, and *Polygonum viviparum*. Findings of this study revealed that *K. myosuroides* excelled in ECM root tip length, dry mass, and NH_4 concentration at -125 °C. Contrarily, *A. nitida* demonstrated the lower values, indicated its minimum tolerance. Notably, *T. repens* boasted the highest nitrogen concentration (18.7 ± 1.31 mg/g), while *P. sylvestris* led in phosphorus (3.2 ± 0.22 mg/g). The *B. pendula* showed the highest potassium concentration (9.4 ± 0.66 mg/g), emphasising species-specific nutrient uptake capabilities in extreme cold conditions. The PCA analysis revealed that the parameters, e.g., NH_4 in soil mix (NH_4), NO_3 in soil mix (NO_3), phosphorus in soil in species of *Polygonum viviparum*, *Kobresia filicina*, *K. myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens* are most accurately represented in cases of + 5 °C, -10 °C, and -20 °C temperatures. On the other hand, the parameters for ECM root tips (ECM) and Dry Mass (DM) are best described in -40 °C, -50 °C, and − 125 °C temperatures. All parameters have a strong influence on the variability of the system indicated the efficiency of ECM. The heatmap supported the nutrients positively correlated with ECM colonization with the host plants.

*Correspondence:
Aisha Umar
ash.dr88@gmail.com

Full list of author information is available at the end of the article

Conclusion At lower temperatures, hyphae and spores in roots were reduced, while soluble phosphorus concentrations of leaves were increased in cold stress soils. Maximum foliar nutrient concentrations were found in *K. myosuroides* at the lowest temperature treatments due to efficient functioning and colonization of ECM.

Keywords Herbaceous plant, Fungi, Ectomycorrhiza, Hartig net, *Kobresia*, *Polygonum*, Altitude

Introduction

Shrub and tree roots are predominantly colonized by ectomycorrhizal (ECM and ECM-like) fungi, whereas they are rarely found in herbs. There are a few shrubs and herbaceous plants that make ECM colonization important for successional changes, e.g., *Arctostaphylos*, *Bistorta*, *Cistus*, *Helianthemum*, *Kobresia*, and *Salix herbacea* [1]. Ectomycorrhizal (ECM) relationships have changed their life styles in different types of plants and fungi [2]. Mycorrhizal herbs play an important role in maintenance and create ectomycorrhizal banks [3]. ECM roots are dominantly present in the upper organic layers of the soil (nearly 20 cm below the surface) [4, 5]. Ectomycorrhizal associations are found all over the world only in 8,000 groups of plants (about 3% of phanerogams) [1].

Plant species are busy to make the involvement with a huge range of Ascomycota and a few with Zygomycota fungal species [6, 7], while maximum association has been found with Basidiomycota [8]. Most of the ECM fungi can live on a wide range of host plants [9], but response is different for different plant species [10]. Vegetative mycelia spread out from the rhizosphere and act as a mycorrhizal inoculum for the nearby host plants [11] via connections with different plant species through a mycorrhizal network.

ECM are important for the natural growth, establishment, and restoration of woody plants, especially in areas damaged by human activities [12]. ECM associations play a prominent role in shaping the structure and movement of ECM plants and forest communities because they affect the soil environment and relationships between the plants [13]. ECM fungi are important for their plant partners for movement into a new area to start the successional processes and growth of plant communities [13]. ECM fungi can change themselves with the passage of time for individual roots, plant communities, and ecosystems as well [5]. Depending on the stage of succession and environmental history, ECM associations provide the different ecological functions and services [14]. ECM fungi are very essential for biogeochemical processes and plants to get nutrients [15]. Mountainous alpine and cold areas of Pakistan are hotspots for biodiversity.

A maximum number of valuable exclusive plants and fungal species are growing there. Adams et al. [3] suggested that ECM fungi frequently colonize herbaceous plants. In cold areas of Pakistan, the studies on the ectomycorrhizal relationship of herbaceous plants are limited because mycorrhizal fungi with plants of cold areas are facing limited availability of essential nutrients. Cold areas are restricted in nutrients due to environmental conditions, so plant growth in such areas is limited due to the unavailability of nutrients [16]. For sake of their survival, these plants develop morpho-anatomical adaptations associated with fungi [17]. Fungal symbiotic association is an effective way to improve the supply of plants with nutrients [18]. The favourable reasons for the ECM relationship in plants of cold areas are abiotic stresses, lack of nutrition, and roots distribution pattern in the soil [19].

The uptake of nutrients through mycorrhizae in cold conditions may also be limited due to the inability of AM (arbuscular mycorrhizal) fungus to act well at low temperatures [20]. Cold climates provoke the nutrient demand in the plants, when the nutrient supply is limited in the soil [20, 21]. The ECM-plants symbiotic relationship is believed to be an effective association in alleviation of nutritional stress for both fungi and plants [22].

The organisms, which are adjusted in adverse conditions, tend to have a greater tolerance to the critical environmental factors than the species actually encounter in their habitats. The continuous exposure of organisms to low temperatures and freezing tolerance is usually fatal for the organism's survival [23]. In addition to ECM fungi, soil microbe communities are affected by the soil moisture and temperature, which leads to nutrient fluctuations. Frost affects the soil nutrient supply by physical breakage of organisms [24].

Here, we tested the hypothesis that ECM are more tolerant towards freezing temperatures and affect the availability of soil nutrients for plants. Moreover, in this study, different plant species were selected from such areas, where cold stress was more dominant, and examined the ectomycorrhizal relationship in the roots of plants along the nutrient's uptake.

Materials and methods

Sampling sites and plants collection

In this study, mycorrhizal associations of *Polygonum viviparum*, *Kobresia filicina*, *K. myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens* were studied. *Kobresia* and *Polygonum* species from Deosai plains of Gilgit-Baltistan, while *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens* were collected from Hazara, Swat, Dir, and Bajaur,

respectively. In this study, selected plant root samples were removed from the depth of 0–15 cm of soil profile.

Morpho-anatomical mycorrhizal colonization

To determine whether the plants have ectomycorrhizal fungi, samples of root were taken from each plant species according to Baker [25]. Plant seedlings collected through the soil cores and ECM roots were washed with distilled water to remove soil particles. Heat treatment for one day of soil killed the endomycorrhizal fungi at 37 °C because ECM fungi were more competitive at lower temperatures [26]. Two subsamples of large root systems (3.0 cm and 6.5 cm depths) of at least 150 root tips from the selected plants per treatment per species were taken. The smallest root systems were needed to be fully observed and finally cut the roots into very small lengths. Clear samples were kept overnight in 10% KOH, 20 min in alkaline hydrogen peroxide, and 2 h in 1% hydrochloride before staining in a solution of glycerol, lactic acid, and methyl blue [27]. The ECM fungal contents of fine sieved roots were examined by dissection method [28] via MX4300H compound light microscope (Meiji Techo Co., Ltd., Japan). Data on anatomical features were recorded.

Turgid, healthy, ectomycorrhiza was fixed in 2% glutaraldehyde in 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 6.5) for four hours, rinsed 5 to 7 times in buffer, dehydrated in ethanol, and embedded in gelatin capsules immediately after dehydration. Thin sections (1–1.2 mm) were cut into MT microtomes, stained for light microscopy with a solution of 1% (w/v) methylene blue, 1% (w/v) azure B, and 1% (w/v) sodium tetraborate in distilled water, and finally rinsed in distilled water. These were counterstained in 0.5% (w/v) basic fuchsin, air dried, mounted in immersion oil, and then observed under a photo microscope.

Soil preparation

The collected soil samples were sieved (6 mm) and homogenized by mixing the volume proportion of 1:2 (meadow soil:soil near the stands) to get soil that contained ECM inoculum. The mixed soil (4 cm thick layer) was placed in aluminium trays and each tray was placed under 6 temperature treatments (control+5 °C, -10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C). Temperature lowered first to +5 °C within 1 h and then decreased to 5 °C/h. Except that temperature was kept for 8 h at -3 °C before further lowering it to the target temperature (for 5 h) to ensure the soil was frozen. Subsequently, the temperature raised again at the rate of 5 °C/h.

At the end, the temperature was kept at +5 °C for 1 h before chamber opening. At -125 °C, the samples were first cooled to -50 °C with the above-described procedure in the same freezing chambers, similarly with other treatments. Afterward the temperature decreased to -125 °C and then increased swiftly to -50 °C: 10 °C h^{-1} in a "liquid-nitrogen cooled chamber (GCC30, Carbolite, Chelmsford, UK)". The soil was then warmed from -50 to +5 °C (Arctest Chamber) at the rate of 5 C h^{-1}. Treated soils and perlite (volume proportion of 30:70) were blended within each treatment.

Pot experiment

Plastic pots with 80 g of soil (water content 30% of dry matter) collected from the root zones of plant species in the filling volume of 185 mL were used. The pot height 80 mm, top diameter 60 mm and base diameter 48 mm. The five seeds of each plant species per treatment were grown in individual pots. Fifteen extra pots without seeds per treatment were left to check the treatments effect on the soluble nutrient concentrations in the soil. Thus, there were 5 (treatments) × 7 (plant species) × 10 (plant individuals) pots were placed in the growth room in randomized arrangement. Surface sterilized seeds of each plant were washed overnight under tap water before sowing. The next day, Tween 80 (one drop) was added and the seeds were shaken for 5 min. Next, seeds were soaked in 30% H_2O_2 for 20 min and were rinsed 5 times in distilled water and 5 seeds were placed in each pot. Covered the seeds in a thin soil layer and germinated in a growth chamber under fluorescent tubes (VHO 215 W, Sylvania Cool White, Sylvania, USA); relative humidity (90%), 22 °C, 20 h day, 4 h night." After germination, the growth conditions were 20 h day at 22 °C, 60% relative humidity, and 4 h night at 17 °C in 80% relative humidity. The cooling/warming rate was 5 °C/h. Day/night irradiance was ca. 350 μmol $m^{-2}s^{-1}$ PAR from incandescent lamps and tubes. Light intensity was changed stepwise in the beginning and end of a day for 2 h.

At the start of 6.5 weeks, the seedlings were thinned to one (per pot), and 4.5 weeks after the sowing, fertilizer N-P-K was applied with all other nutrients. By this, 'N' dose per week was 1 mg in 10 mL water. Watered all pots till those reached the 60% water holding capacity, and plants were harvested 11 weeks after the sowing.

Soil properties

Without frost treatments of the soil mixture, dry matter content (105 °C) was estimated. For water holding capacity (of soil mixture): weighed out the five wet filter papers and placed in funnels (equipped with rubber tubes and pinch clamps), where 5 g soil mixture in 100 mL water was presented. For 2 h, soils were incubated, and after that, slightly clamps opened until the water flow ceased (ca. 30 min). Soil saturated with water was weighed out. The soil samples were dried at 105 °C and weighed out again. The water holding capacity of the soil was calculated (per dry matter). Dry (40 °C) biomass of seedling parts was also estimated.

Plant nutrients

Total nitrogen concentration of initial soil, soil mix, and plants was determined by Kjeldahl method [30]. The other nutrients were determined by "ICP-OES (Iris Intrepid II XSP, Thermo Elemental, Franklin, MA, USA)" after MARS5 microwave wet digestion in HNO_3 and H_2O_2 in Teflon containers. For soil nutrient analysis, 75 pots were watered like other pots during the growing period without fertilization. At the same time, soil was sampled with plant harvest, dried (40 °C), and dried matter content (105 °C) was determined. Soil (10 g) was mixed in 1 M KCl (100 mL) for 1 h. Filtered the extracts, and NO_3-N and NH_4-N concentrations were calculated by the flow injection analyzer "(FIAstar 5012, Tecator, Sweden)". Soil of the 75 pots was analyzed for other nutrients (ICP-OES after ammonium acetate extraction) at pH 4.65.

Statistical analysis

A one-way ANOVA was used to compare numeric variables across different groups. Post-hoc comparisons using the Least Significant Difference (LSD) were conducted to determine the pairwise differences. If the difference between two means was greater than the LSD value, that difference was considered statistically significant at a level of 0.05. The significance level (α) was set at 0.05, indicated a *p*-value less than 0.05 considered statistically significant. SPSS 28 IBM software for Windows was used for the statistical analysis.

Principal component analysis was used to determine the relationships between the studied cases and parameters. Statistical software (version 12.0, StatSoft Inc., Tulsa, OK, USA) was used for statistical analysis. Principal components analysis (PCA) was performed at the significance level, and the matrix of data used for the PCA statistical analysis of the temperature test resulted in 7 rows and 36 columns for ECM root tips: Dry Mass (DM), NH_4 in soil mix (NH_4), NO_3 in soil mix (NO_3), and Phosphorus in soil (P). The matrix for the statistical analysis of chemical elements had 7 rows and 15 columns for concentrations and for content of nutrients in plant leaves. The optimal number of main components obtained in both cases was determined based on the Cattel criterion. The input matrix has been automatically rescaled.

Results

Cold stressed plants and ECM colonization

The species of plants are commonly found in cold alpine and stress-inducing habitats. Fungus made anatomical structures with *K. filicina* (Fig. 1A), *K. myosuroides* (Fig. 1B), and *P. viviparum* (Fig. 1C) herbaceous plant species of stress-making habitats. In this study, *K. filicina*, *K. myosuroides*, and *P. viviparum* showed the ECM associations with ascomycete fungus (*Cenococcum geophilum)* rather than endomycorrhizal type (Fig. 1D). Endomycorrhizal type was absent in these targeted species. Spherical cleistothecia of *C. geophilum* was covered by the dark pigmented hyphae (groups of isodiametric cells) encircled by elongated cells in a stellate pattern. A few parts of cleistothecia were covered by emanating hyphae with rounded bases, and a few rounded cells encapsulated by hyphae (gelatinous in wet but hard when dry) in a dense network form (Fig. 1D1). The stiff, simple, septate, and without clamp connections hyphae of the ECM fungus were found in these plant species (Fig. 1D2).

Cells of mantle formed by fungal species were extended by bristle-like hyphae (two-layered and thick-walled). The extended bristle-like hyphae were septate, while simple pores were seen in the hyphae of the mantle and Hartig net. Mantle cells were compactly arranged without intercellular spaces. Fungal cells were penetrated longitudinally in the outer cortical cells of the root and then into the inner cells of the root to form the Hartig net. The hyphal walls of Hartig net were thin, without intercellular spaces, uniform, and uniformly contacted the adjacent walls of cortical cells (Fig. 1D1, D2).

Specialized dauciform roots were seen in *K. filicina* (Fig. 2A). These roots are colonized by ECM fungus, facilitating the supply of nutrients from the nutrients deficient soil. The shape of the dauciform roots resembled the carrots, and its surface was free from root hairs (Fig. 2A1). In this study, dauciform roots were beige in colour. The originating pattern of these roots was lateral and appeared like a peduncle or non-peduncle from the main plant root. Hartig net was not seen in this root type (Fig. 2A1). Ectomycorrhiza of *Cenococcum geophilum* in *K. myosuroides* (Fig. 2B) formed the epidermal Hartig net and fungal mantle (Fig. 2B1). Hyphae of Hartig net were radially elongated in root epidermal cells, and the mantle formed by many layers of compactly organized hyphae.

Polygonum viviparum was strongly influenced by the ECM fungal colonization (Fig. 2C). Simple, club-shaped, cylindrical, and bright-coloured ectomycorrhizal fungal hyphae were observed in *P. viviparum*. The *P. viviparum* roots were a network of several mycorrhizal lateral roots (both short and long) that emerged from the plant's tiny propagules (bulbils generated in cold conditions). Due to different *Cenococcum* species, some ECM have light-coloured mantles, while others have dark-coloured mantles. The mantle (Fig. 2C1) covered the root apex and initiated a distinct epidermal Hartig net near the apical meristem. A variety of ECM has polysaccharide-rich mantles. The root cap was reduced by the plants' interaction with fungal species. Hartig net cells were penetrated in the single elongated layer of root epidermal cells (Fig. 2C2).

Cenococcum fungus happily associated the *K. myosuroides*, *K. filicina*, and *P. viviparum.* Mantle texture and

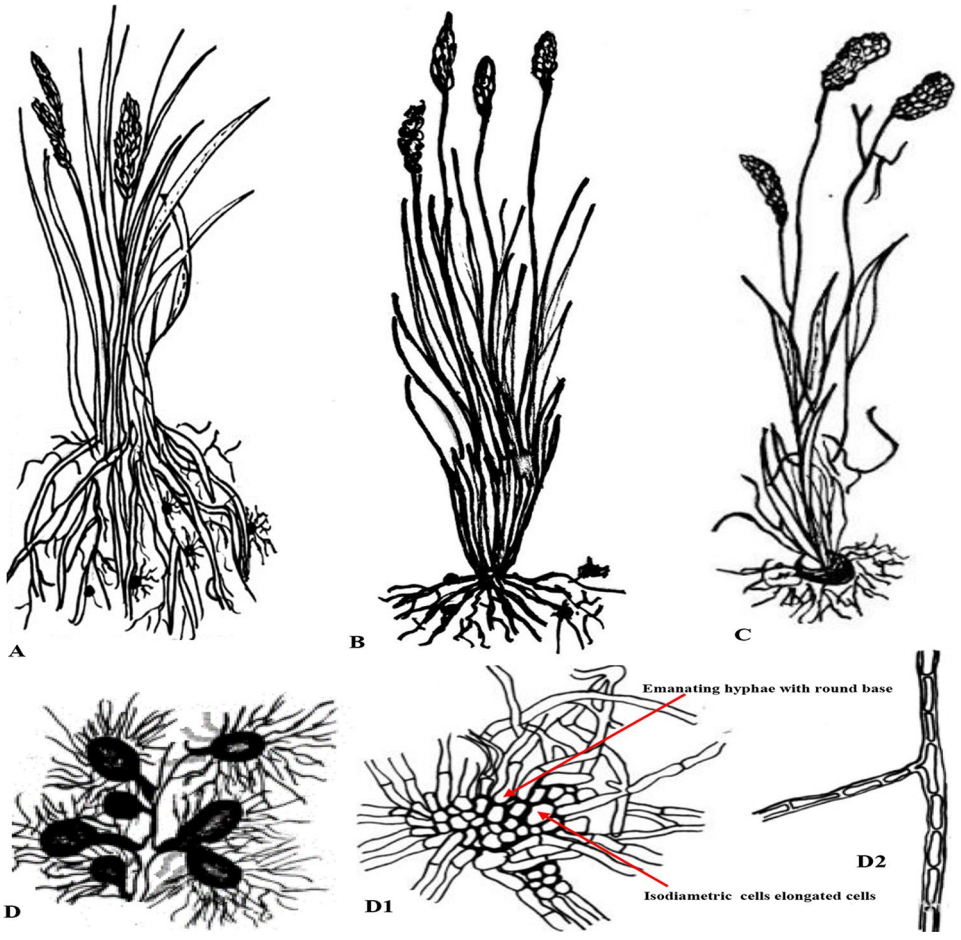

Fig. 1 Plant root system with ECM fungal species **A.** *Kobresia filicina*, ***B.*** *Kobresia myosuroides*, **C.** *Polygonum viviparum*, and **D.** Mycelium of *Cenococcum geophilum* (**D1**. Spherical cleistothecia **D2**. Thick-walled mycelium, two-layered)

colour of *K. myosuroides* and *P. viviparum* depended on ECM morphotypes. The orchestration of ECM fungal anatomical structures depends upon the hyphal division, behaviour, rearrangements, and cell shape.

Effects of temperature on ECM roots

The length of ECM root tips was measured under different temperature conditions (-10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C) and compared to a control temperature of 5 °C. Statistical analysis, including mean and standard deviation calculations were performed. The differences in mean ECM root tip length between the temperature conditions were evaluated using the LSD (Least Significant Difference) test. The length of ECM root tips varied among the different temperature conditions and plant species. At the control temperature of 5 °C, *Polygonum viviparum* had a mean ECM root tip length of 40.0±2.8 mm. The length increased to 52.0±3.6 mm at -10 °C, 54.0±3.8 mm at -20 °C, 57.0±4.0 mm at -30 °C, 60.0±4.2 mm at -40 °C, 62.0±4.3 mm at -50 °C, and 64.0±4.5 mm at -125 °C. If the difference between two means was greater than the LSD value, the difference was considered statistically significant at a level of 0.05 (Table 1).

Similarly, other plant species, such as *Kobresia filicina*, *K. myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens*, showed variations in mean ECM root tip length across different temperature conditions. The mean ECM root tip lengths generally increased with decreasing temperature.

Effects of temperature on dry mass

The dry mass measurements were taken at different temperature conditions (-10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C) and compared to a control temperature of 5 °C. The dry mass of the plant species exhibited variations under different temperature conditions. For *Polygonum viviparum*, the lowest mean dry mass was observed at the control temperature (5 °C) with a value of 2±0.14 g. It increased to 2.2±0.15 g at -10 °C, 2.4±0.17 g at -20 °C, 2.6±0.18 g at -30 °C, 2.8±0.2 g at -40 °C, 3±0.21 g at -50 °C, and 3.2±0.22 g at -125 °C.

The standard deviations (SD) for these measurements ranged from 0.13 to 0.22 g. The LSD analysis indicated

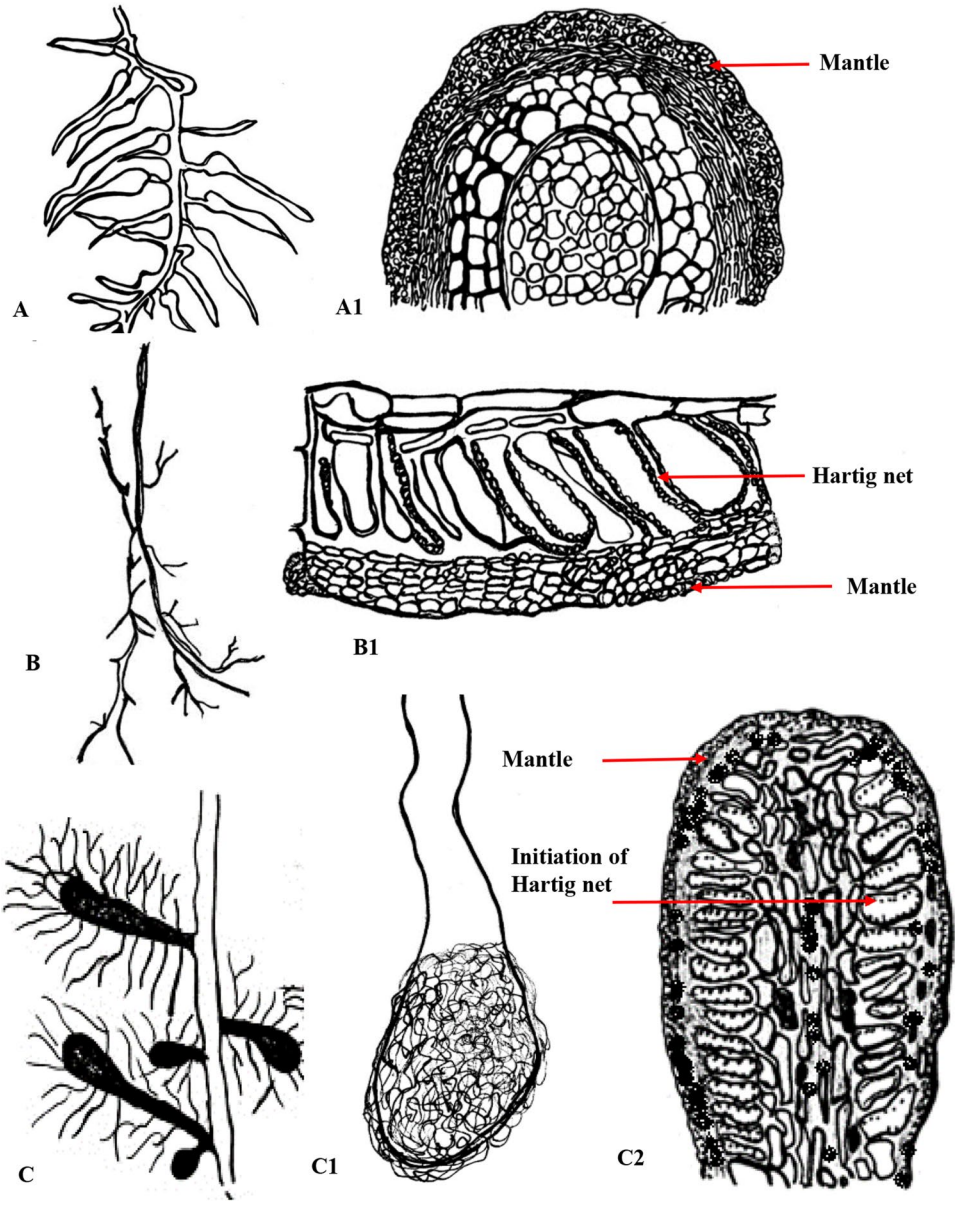

Fig. 2 (**A**) Dauciform roots of *K. filicina*. **A1.** Transverse section of a dauciform root with mantle. (**B**) Mycorrhizal roots of *K. myosuroides*. **B1.** Arrow indicated compact mantle and radially enlarged epidermal cells and well-developed Hartig net of *K. myosuroides*. (**C**) Mycorrhizal roots of *P. viviparum*. **C1.** Dark mycorrhizal fungal mantle of *P. viviparum*. **C2.** Two-layered ectomycorrhiza mantle (arrowed), initiation of Hartig net (arrowed), and radially elongated epidermal cells (red arrowed) of *P. viviparum*

that the differences in mean dry mass between the control temperature and -10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C were statistically significant, with LSD values of 0.61, 0.25, 0.13, 0.02, 0.18, and 0.15, respectively ($p<0.05$).

Kobresia filicina also showed variations in mean dry mass across different temperature conditions. The mean dry mass was lowest at the control temperature (5 °C) with a value of 1.1±0.08 g. It increased to 1.5±0.11 g at -10 °C, 2.2±0.15 g at -20 °C, 3±0.21 g at -30 °C, 3.5±0.25 g at -40 °C, 3.8±0.27 g at -50 °C, and 4.1±0.29 g at -125 °C.

The standard deviations for these measurements ranged from 0.08 to 0.29 g. The LSD test revealed that the differences in mean dry mass between the control temperature and -10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C were statistically significant, with LSD values of 0.61, 0.25, 0.13, 0.02, 0.18, and 0.15, respectively ($p<0.05$) (Table 2). Similar trends were observed for the other plant species, *Kobresia myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens.*

Table 1 Effects of temperature on ECM Root tip length in different plant species

Temp	(ECM root tips)						
	Polygonum viviparum	*Kobresia filicina*	*Kobresia myosuroides*	*Alnus nitida*	*Betula pendula*	*Pinus sylvestris*	*Trifolium repens*
5 °C	40.0 ± 2.8	65.0 ± 5.2	60.0 ± 4.2	29.0 ± 2.0	52.0 ± 3.6	40.0 ± 2.8	30 ± 2.4
-10 °C	52.0 ± 3.6	70.0 ± 4.9	62.0 ± 4.3	32.0 ± 2.2	53.0 ± 4.2	43.0 ± 3.0	32 ± 2.2
-20 °C	54.0 ± 3.8	72.0 ± 6.5	65.0 ± 4.6	34.0 ± 2.7	54.0 ± 3.8	45.0 ± 3.2	34 ± 1.7
-30 °C	57.0 ± 4.0	75.0 ± 6.0	67.0 ± 4.7	35.0 ± 2.8	54.0 ± 3.2	48.0 ± 3.4	36 ± 2.9
-40 °C	60.0 ± 4.2	78.0 ± 4.7	69.0 ± 4.8	38.0 ± 2.7	57.0 ± 4.0	49.0 ± 3.4	38 ± 2.7
-50 °C	62.0 ± 4.3	80.0 ± 6.4	70.0 ± 4.9	40.0 ± 2.8	59.0 ± 4.7	50.0 ± 3.5	39 ± 2.7
-125 °C	64.0 ± 4.5	82.0 ± 5.7	75.0 ± 5.3	42.0 ± 2.9	60.0 ± 4.2	52.0 ± 3.6	40 ± 3.2
LSD	**3.89**	**4.08**	**3.16**	**2.21**	**2.61**	**2.2**	**2.54**

Results are (means ± S.D.) ($n = 3$), *Significance difference of ECM root tips for each plant species at different cold temperatures p-value < 0.05. Control temperature (5 °C)

Table 2 Effects of temperature on dry mass of the plant species in soil mix

Temp	Dry mass						
	Polygonum viviparum	*Kobresia filicina*	*Kobresia myosuroides*	*Alnus nitida*	*Betula pendula*	*Pinus sylvestris*	*Trifolium repens*
5 °C	2 ± 0.14	1.1 ± 0.08	1.3 ± 0.09	0.5 ± 0.04	0.2 ± 0.01	0.4 ± 0.03	1.5 ± 0.11
-10 °C	2.2 ± 0.15	1.5 ± 0.11	1.4 ± 0.1	0.7 ± 0.05	0.3 ± 0.02	0.5 ± 0.04	1.4 ± 0.1
-20 °C	2.4 ± 0.17	2.2 ± 0.15	1.6 ± 0.11	0.9 ± 0.06	0.5 ± 0.04	0.6 ± 0.04	1.2 ± 0.08
-30 °C	2.6 ± 0.18	3 ± 0.21	1.8 ± 0.13	0.8 ± 0.06	0.7 ± 0.05	0.7 ± 0.05	1.1 ± 0.08
-40 °C	2.8 ± 0.2	3.5 ± 0.25	2 ± 0.14	1 ± 0.07	0.8 ± 0.06	0.7 ± 0.05	0.9 ± 0.06
-50 °C	3 ± 0.21	3.8 ± 0.27	2.8 ± 0.2	1.5 ± 0.11	0.9 ± 0.06	0.8 ± 0.06	0.5 ± 0.04
-125 °C	3.2 ± 0.22	4.1 ± 0.29	2.9 ± 0.2	2 ± 0.14	1 ± 0.07	0.9 ± 0.06	0.4 ± 0.03
LSD	**0.61**	**0.25**	**0.13**	**0.02**	**0.18**	**0.11**	**0.15**

Results are (means ± S.D.) ($n = 3$), *Significance difference of Dry Mass for each plant species at different cold temperatures p-value < 0.05. Control temperature (5 °C)

Table 3 Effects of temperature on NH_4 concentrations in soil mix

Temp.	NH_4 in soil mix						
	Polygonum viviparum	*Kobresia filicina*	*Kobresia myosuroides*	*Alnus nitida*	*Betula pendula*	*Pinus sylvestris*	*Trifolium repens*
5 °C	70 ± 4.9	60 ± 4.2	80 ± 5.6	20 ± 1.4	50 ± 3.5	45 ± 3.2	40 ± 2.8
-10 °C	65 ± 4.6	58 ± 4.1	75 ± 5.3	18 ± 1.3	45 ± 3.2	43 ± 3	38 ± 2.7
-20 °C	56 ± 3.9	56 ± 3.9	64 ± 4.5	15.6 ± 1.1	42 ± 2.9	40 ± 2.8	37.8 ± 2.6
-30 °C	52 ± 3.6	55 ± 3.9	60 ± 4.2	15 ± 1.1	40 ± 2.8	37 ± 2.6	37 ± 2.6
-40 °C	50 ± 3.5	40 ± 2.8	58 ± 4.1	14 ± 1	25 ± 1.8	30 ± 2.1	45 ± 3.2
-50 °C	42 ± 2.9	32 ± 2.2	55 ± 3.9	12 ± 0.8	22 ± 1.5	24 ± 1.7	32 ± 2.2
-125 °C	40 ± 2.8	30 ± 2.1	50 ± 3.5	15 ± 1.1	20 ± 1.4	20 ± 1.4	30 ± 2.1
LSD	**2.7**	**3.12**	**3.48**	**1.19**	**2.3**	**3.18**	**2.7**

Results are (means ± S.D.) ($n = 3$), *Significance difference of NH4 in soil mix for each plant species at different cold temperatures p-value < 0.05. Control temperature (5 °C)

The mean dry mass generally increased with decreasing temperature.

Effects of temperature on ammonium (NH_4) concentrations in soil mix

This study aimed to examine the effects of different temperature conditions on ammonium concentrations in a soil mix. The mean ammonium concentrations were determined for various plant species (*Polygonum viviparum*, *Kobresia filicina*, *K. myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens*) under different temperature conditions (-10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C). The mean ammonium concentrations varied among the temperature conditions for each plant species. For *Polygonum viviparum*, the highest mean ammonium concentration was observed at the control temperature (5 °C) with a value of 70 ± 4.9 mg/kg. It decreased to 65 ± 4.6 mg/kg at -10 °C, 56 ± 3.9 mg/kg at -20 °C, 52 ± 3.6 mg/kg at -30 °C, 50 ± 3.5 mg/kg at -40 °C, 42 ± 2.9 mg/kg at -50 °C, and 40 ± 2.8 mg/kg at -125 °C.

The standard deviations (SD) for these measurements ranged from 2.1 to 4.9. The LSD analysis revealed that the differences in mean ammonium concentrations between the control temperature and −10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C were statistically significant, with LSD values of 2.7, 3.12, 3.48, 1.19, 2.3, and 2.7, respectively ($p < 0.05$) (Table 3).

Kobresia filicina also exhibited variations in mean ammonium concentrations across different temperature conditions. The mean ammonium concentration was highest at the control temperature (5 °C) with a value of 60±4.2 mg/kg. It decreased to 58±4.1 mg/kg at -10 °C, 56±3.9 mg/kg at -20 °C, 55±3.9 mg/kg at -30 °C, 40±2.8 mg/kg at -40 °C, 32±2.2 mg/kg at -50 °C, and 30±2.1 mg/kg at -125 °C.

The standard deviations for these measurements ranged from 2.1 to 4.2. The LSD test indicated that the differences in mean ammonium concentrations between the control temperatures, -10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C were statistically significant, with LSD values of 2.7, 3.12, 3.48, 1.19, 2.3, and 2.7, respectively ($p<0.05$) (Table 3). Similar patterns were observed for the other plant species, *Kobresia myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens.* The mean ammonium concentrations decreased with decreasing temperature.

Effects of temperature on nitrate (NO_3) concentrations in soil mix

The effects of different temperature conditions on nitrate concentrations in a soil mix were determined in this work. The mean nitrate concentrations were measured for various plant species under different temperature conditions (-10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C). The mean nitrate concentrations varied among the temperature conditions for each plant species. For *Polygonum viviparum*, the mean nitrate concentration was highest at the control temperature (5 °C) with a value of 50 mg/kg, and it decreased to 45 mg/kg at -10 °C, 42 mg/kg at -20 °C, 40 mg/kg at -30 °C, 25 mg/kg at -40 °C, 22 mg/kg at -50 °C, and 20 mg/kg at -125 °C. The standard deviations (SD) for these measurements ranged from 1.4 to 3.5. The LSD analysis revealed that the differences in mean nitrate concentrations between the control temperature and -10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C were statistically significant, with LSD values of 2.31, 2.51, 2.54, 2.84, 1.58, and 1.7, respectively. *Kobresia filicina* also exhibited variations in mean nitrate concentrations across different temperature conditions. The mean nitrate concentration was highest at the control temperature (5 °C) with a value of 40 mg/kg. It decreased to 37 mg/kg at -10 °C, 36 mg/kg at -20 °C, 33.5 mg/kg at -30 °C, 33 mg/kg at -40 °C, 32 mg/kg at -50 °C, and 30 mg/kg at -125 °C. The standard deviations for these measurements ranged from 2 to 3.2. The LSD test indicated that the differences in mean nitrate concentrations between the control temperature and -10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C were statistically significant, with LSD values of 2.31, 2.51, 2.54, 2.84, 1.58, and 1.7, respectively ($p<0.05$) (Table 4).

Effects of temperature on Phosphorus concentrations in soil

The mean phosphorus concentrations were measured for plant species (*Polygonum viviparum*, *Kobresia filicina*, *K. myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens*) under different temperature conditions (-10 °C, -20 °C, -30 °C, -40 °C, -50 °C, and -125 °C). Statistical analysis, including one-way analysis of variance (ANOVA) and post-hoc tests was performed to assess the significance of the differences in mean phosphorus concentrations among the temperature conditions. The mean phosphorus concentrations varied among the temperature conditions for each plant species. For *Polygonum viviparum*, the mean phosphorus concentration was 14 mg/kg at control temperature (5 °C), but it decreased to 12 mg/kg at -10 °C and further declined to 8.5 mg/kg at -40 °C. The LSD analysis revealed that the difference between the mean phosphorus concentrations at control temperature and -10 °C was statistically significant (LSD=1.73, $p<0.05$). Similarly, a significant difference was observed between the mean phosphorus concentration at control temperature and -40 °C (LSD=1.73, $p<0.05$). *Kobresia filicina* exhibited a different pattern, with the mean phosphorus concentration being 14 mg/kg at control temperature, 11 mg/kg at -10 °C, and 6 mg/kg at -50 °C. The LSD test indicated that

Table 4 Effects of temperature on nitrate concentrations in soil mix

Temp.	NO_3 in soil mix						
	Polygonum viviparum	*Kobresia filicina*	*Kobresia myosuroides*	*Alnus nitida*	*Betula pendula*	*Pinus sylvestris*	*Trifolium repens*
5 °C	50±3.5	40±3.2	45±3.2	40±2.8	20±1.4	30±2.1	20±1.6
-10 °C	45±3.2	37±2.6	43±3	38±2.7	18±1.4	18±1.3	19±1.3
-20 °C	42±2.9	36±3.2	40±2.8	37.8±3	15.6±1.1	15.6±1.2	18.9±1.9
-30 °C	40±2.8	33.5±2.7	37±2.6	37±3	15±2.3	15±1.3	18±1.4
-40 °C	25±1.8	33±2	30±2.1	45±3.2	14±1.6	14±1.4	16±1.6
-50 °C	22±1.5	32±2.6	24±1.7	32±2.2	12±1.9	12±1.8	15.5±1.8
-125 °C	20±1.4	30±2.1	20±1.4	30±2.1	15±1.3	20±1.4	15±1.2
LSD	**2.31**	**2.51**	**2.54**	**2.84**	**1.58**	**1.7**	**1.54**

Results are (means±S.D.) ($n=3$), *Significance difference of NO_3 in soil mix for each plant species at different cold temperatures p-value<0.05. Control temperature (5 °C)

Table 5 Effects of temperature on phosphorus concentrations in soil

Temp.	Phosphorus in soil						
	Polygonum viviparum	*Kobresia filicina*	*Kobresia myosuroides*	*Alnus nitida*	*Betula pendula*	*Pinus sylvestris*	*Trifolium repens*
5 °C	14 ± 0.98	14 ± 0.98	16 ± 1.12	10 ± 0.7	15 ± 1.05	12 ± 0.84	10 ± 0.7
-10 °C	12 ± 0.84	11 ± 0.77	15 ± 1.05	8.9 ± 0.62	10.5 ± 0.74	10 ± 0.7	9 ± 0.63
-20 °C	11 ± 0.77	10 ± 0.7	14.5 ± 1.02	7.5 ± 0.53	9 ± 0.63	9.6 ± 0.67	8.6 ± 0.6
-30 °C	9 ± 0.63	7.8 ± 0.55	14 ± 0.98	8 ± 0.56	8 ± 0.56	9 ± 0.63	7.5 ± 0.53
-40 °C	8.5 ± 0.6	6 ± 0.42	12 ± 0.84	6.5 ± 0.46	7 ± 0.49	8.4 ± 0.59	7 ± 0.49
-50 °C	7 ± 0.49	5 ± 0.35	9 ± 0.63	9.9 ± 0.69	6.5 ± 0.46	8 ± 0.56	6.8 ± 0.48
-125 °C	5 ± 0.35	3 ± 0.21	8 ± 0.56	7 ± 0.49	6 ± 0.42	6 ± 0.42	5 ± 0.35
LSD	**1.73**	**1.23**	**2.17**	**1.35**	**1.39**	**1.71**	**1.02**

Results are (means ± S.D.) ($n = 3$), *Significance difference of Phosphorus in Soil for each plant species at different cold temperatures p-value < 0.05. Control temperature (5 °C)

Table 6 Nutrients concentrations in plant leaves of different species at -125 °C

	Mean conc. (mg g^{-1}) of nutrients in plants leaves at -125 °C							LSD
	Polygonum viviparum	*Kobresia filicina*	*Kobresia myosuroides*	*Alnus nitida*	*Betula pendula*	*Pinus sylvestris*	*Trifolium repens*	
N	20 ± 1.40	16 ± 1.12	15.5 ± 1.09	17.7 ± 1.24	11 ± 0.77	14.6 ± 1.02	18.7 ± 1.31	1.14
P	2.5 ± 0.18	1.5 ± 0.11	2.1 ± 0.15	1.4 ± 0.10	2.4 ± 0.17	3.2 ± 0.22	2.2 ± 0.15	1.02
K	8.7 ± 0.61	7.7 ± 0.54	6.5 ± 0.46	8.6 ± 0.60	9.4 ± 0.66	7.4 ± 0.52	8.5 ± 0.60	2.57
Ca	5.4 ± 0.38	8.5 ± 0.60	3.5 ± 0.25	4.6 ± 0.32	4.5 ± 0.32	4.8 ± 0.34	4.6 ± 0.32	1.36
Cu	4.7 ± 0.33	4.8 ± 0.34	7.3 ± 0.51	6.5 ± 0.46	3.8 ± 0.27	8.5 ± 0.60	7.6 ± 0.53	1.72
Mn	300 ± 5.00	97. 5 ± 6.83	205 ± 4.60	150 ± 6.50	400 ± 7.5	200 ± 4.90	160 ± 4.20	14.27
Zn	200 ± 4.30	38 ± 2.66	150 ± 5.20	70 ± 4.90	180 ± 6.2	170 ± 6.30	76 ± 5.32	9.57

Results are (means ± S.D.) ($n = 3$), *Significance difference of nutrients concentrations in each plant leaves species at forest temperature (-125 °C), p-value < 0.05

the difference between the mean phosphorus concentrations at control temperature and -10 °C was statistically significant (LSD = 1.23, $p < 0.05$). Likewise, a significant difference was found between the mean phosphorus concentration at control temperature and -50 °C (LSD = 1.23, $p < 0.05$) (Table 5).

The other plant species (*Kobresia myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens*) also showed variations in mean phosphorus concentrations across different temperature conditions. However, the differences were not statistically significant based on the LSD values calculated at a significance level of 0.05 (LSD values ranging from 1.02 to 2.17).

Nutrients concentrations in plant leaves

This study aimed to investigate the nutrient concentrations in the leaves of various plant species at an extreme cold temperature (-125 °C). The mean concentrations and standard deviations (SD) of nutrients were measured for seven plant species, including *Polygonum viviparum*, *Kobresia filicina*, *K. myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens.* The statistical significance of the observed differences was evaluated using a significance level of 0.05. The results revealed the variations in nutrient concentrations among the different plant species at -125 °C. Each nutrient showed distinct patterns across the species.

Nitrogen (N) in *P. viviparum* exhibited the highest mean concentration (1.40 mg g^{-1}), while *B. pendula* showed the lowest (11 mg g^{-1}) concentration. If the difference in mean 'N' concentrations between two plant species greater than 1.14, the difference was considered statistically significant at a significance level of 0.05 (Table 6).

Phosphorus (P) concentrations ranged from 1.4 to 3.2 mg g^{-1}, with *K. filicina* having the lowest mean concentration (1.5 mg g^{-1}) and *P. sylvestris* having the highest (3.2 mg g^{-1}) values. If the difference in mean 'P' concentrations between two plant species was greater than 1.02, this difference was considered statistically significant at a significance level of 0.05.

Potassium (K) concentrations varied from 6.5 to 9.4 mg g-1, with *K. myosuroides* having the lowest mean concentration (6.5 mg g^{-1}) and *B. pendula* having the highest (9.4 mg g^{-1}). If the difference in mean 'K' concentrations between two plant species was greater than 2.57, the difference was considered statistically significant at a significance level of 0.05.

Calcium (Ca) concentrations ranged from 3.5 to 8.5 mg g^{-1}, with *K. myosuroides* exhibited the lowest mean concentration (3.5 mg g^{-1}) and *K. filicina* having the highest (8.5 mg g^{-1}). If the difference in mean 'Ca' concentrations between two plant species greater than 1.36, the difference was considered statistically significant at a significance level of 0.05.

Copper (Cu) concentrations varied from 3.8 to 8.5 mg g^{-1}, with *T. repens* exhibited the lowest mean concentration (3.8 mg g^{-1}), while *P. sylvestris* has highest (8.5 mg g^{-1}) concentration. If the difference in mean 'Cu' concentrations between two plant species greater than 1.72, the difference was considered statistically significant at a level of 0.05.

Manganese (Mn) concentrations ranged from 97.5 to 400 mg g^{-1}, *K. filicina* presented the lowest mean concentration (97.5 mg g^{-1}) and *P. viviparum* having the highest (400 mg g^{-1}). If the difference in mean 'Mn' concentrations between two plant species was greater than 14.72, the difference was considered statistically significant at a significance level of 0.05 (Table 6).

Principal component analysis (PCA) for temperature

Performing principal component analysis (PCA) allowed us to obtain six new variables that explained 100% of the variability of the system. The PCA analysis showed that the first two main components PC1 and PC2 explained 93.13% of the variability of the system. All parameters except *Pinus sylvestris* for NO_3 in soil mix have a strong influence on the variability of the system because they fallen between the two red circles (Fig. 3A). This parameter also affects the variability of the system, but it was not as strong as other parameters. The parameters for NH_4 in soil mix (NH_4), NO_3 in soil mix (NO_3), Phosphorus in soil (P): *Polygonum viviparum*, *Kobresia filicina*, *K. myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens* were strongly and positively correlated. *Trifolium repens*, except: NO_3 *Alnus nitida* and NO_3 *Pinus sylvestris* for NO_3 in soil mix (NO_3), NH_4 *Trifolium repens* for NH_4 in soil mix, and 'P' *Alnus nitida* for Phosphorus in soil.

There was also a positive and strong correlation between the parameters for ECM root tips (ECM), Dry Mass (DM): *P. viviparum*, *K. filicina*, *K. myosuroides*, *(A) nitida*, *(B) pendula*, *P. sylvestris*, and *T. repens*. A strong and negative correlation was observed between the parameters for ECM root tips (ECM), Dry Mass (DM), and the parameters for NH_4 in soil mix (NH_4), NO_3 in soil mix (NO_3), Phosphorus in soil (P) except NO_3 *Alnus nitida*, NO_3 *Pinus sylvestris*, NH_4 *Trifolium repens*, 'P' *Alnus nitida*.

The PCA analysis also showed that the first principal component (PC1) described 86.16% of the differences between the parameters for ECM root tips (ECM), Dry Mass (DM) and NH_4 in soil mix (NH_4), NO_4 in soil mix (NO_3), Phosphorus in soil (P) (Fig. 3B). Positive values of the main component PC1 described the results for ECM root tips (ECM), Dry Mass (DM), and negative values of the main component PC1 explained the results parameters for NH_4 in soil mix (NH_4), NO_3 in soil mix (NO_3), and Phosphorus in soil (P). The second principal component (PC2) in 6.97% described the differences between NO_3 *Alnus nitida*, NH_4 *Trifolium repens*, NO_3 *Pinus sylvestris*, and 'P' *Alnus nitida*. Positive values of the main component PC2 described the results for NO_3 *Alnus nitida* and NH_4 *Trifolium repens*, and negative values of the main component PC2 described the results for 'NO_3' *Pinus sylvestris* and 'P' *Alnus nitida*.

The PCA analysis (Fig. 3A, B) indicated that parameters for NH_4 in soil mix, NO_3 in soil mix, phosphorus in soil, *Polygonum viviparum*, *Kobresia filicina*, *K. myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens* were best described by conditions at 5 °C, -10 °C, and -20 °C, while parameters for ECM root tips and dry mass were best described by -40 °C, -50 °C, and -125 °C.

Principal component analysis (PCA) for chemical elements

Performing principal component analysis (PCA) allowed us to obtain six new variables that explained 100% system variability. The PCA analysis showed that the first two main components PC1 and PC2 explained 99.59% of the variability of the system. All parameters have a strong influence on the variability of the system because these all presented in the red circle (Fig. 4A). The parameters for the concentration in mg g^{-1} of nutrients in plant leaves at -125 °C were strongly and positively correlated: *Polygonum viviparum*, *Kobresia filicina*, *K. myosuroides*, *Alnus nitida*, *Betula pendula*, *Pinus sylvestris*, and *Trifolium repens*. There was also a positive and strong correlation for the content in 'mg' of nutrients in plant leaves at -125 °C between the parameters: *P. viviparum*, *K. filicina*, *K. myosuroides*, *(A) nitida*, *(B) pendula*, *P. sylvestris*, and *T. repens*. No correlation was observed between these parameters for the concentration in mg g^{-1} of nutrients in plant leaves at -125 °C, and for the content in 'mg' of the nutrients in plant leaves at -125 °C. A more homogeneous group was formed by the parameters for the content than for the concentration of nutrients in plant leaves at -125 °C.

The PCA analysis exhibited that the first principal component (PC1) described the differences between 'Ca, Cu, K, N, P, Mn, and Zn' in 64.17% (Fig. 4B). Positive values of the PC1 principal component explained the results for Ca, Cu, K, N, and P, and negative values of the PC1 described the results for Mn and Zn. The second principal component (PC2) exhibited the differences between Mn and Zn in 35.43%. Positive values of the main component PC2 described the results for 'Mn', and negative values of the main component PC2 represented the 'Zn'.

The PCA analysis (Fig. 4A, B) also explained that the parameters of *P. viviparum*, *K. filicina*, *K. myosuroides*, *(A) nitida*, *(B) pendula*, *P. sylvestris*, and *T. repens*

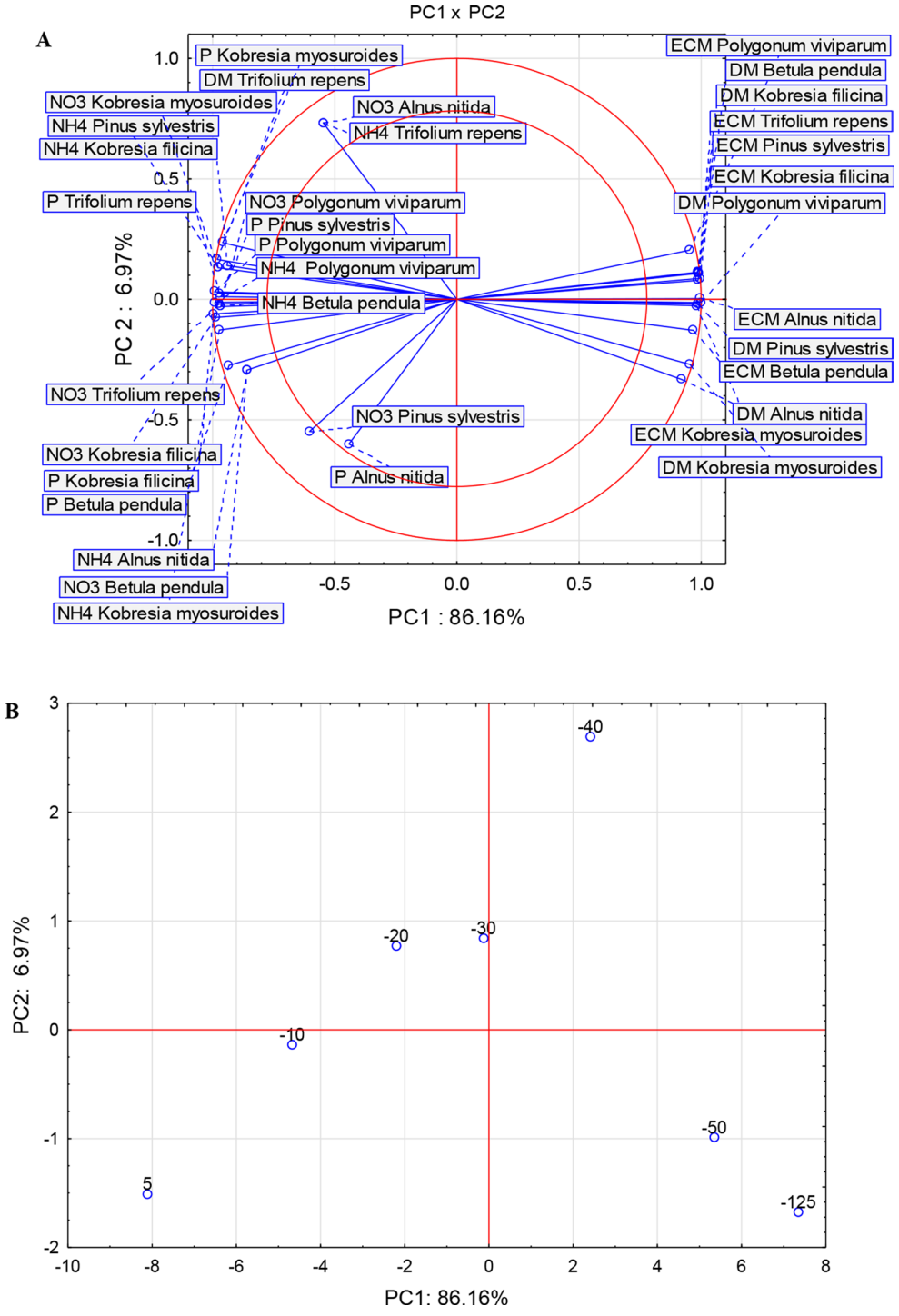

Fig. 3 (**A**) Projection of variables: parameters on the PC1 and PC2 loadings plot, (**B**) Projection of sample type on the PC1 and PC2 scores plot

for concentration in mg g^{-1} of nutrients in plant leaves at -125 °C and best described the 'Mn' and the parameters for the content in mg of nutrients in plant leaves at -125 °C showed excellence for Zn. No significant differences were observed in the concentration and content of parameters in the case of Ca, Cu, K, N, and P.

Correlation

Combinations of pair of variables were mentioned in Table 7 (Table S1) of the heat map. The table's row and column headers comprised the name of the variables and numerical values of the calculated correlation coefficients (inside the tables). The value of the correlation coefficient was closed in the range of -1, 1. The larger its absolute

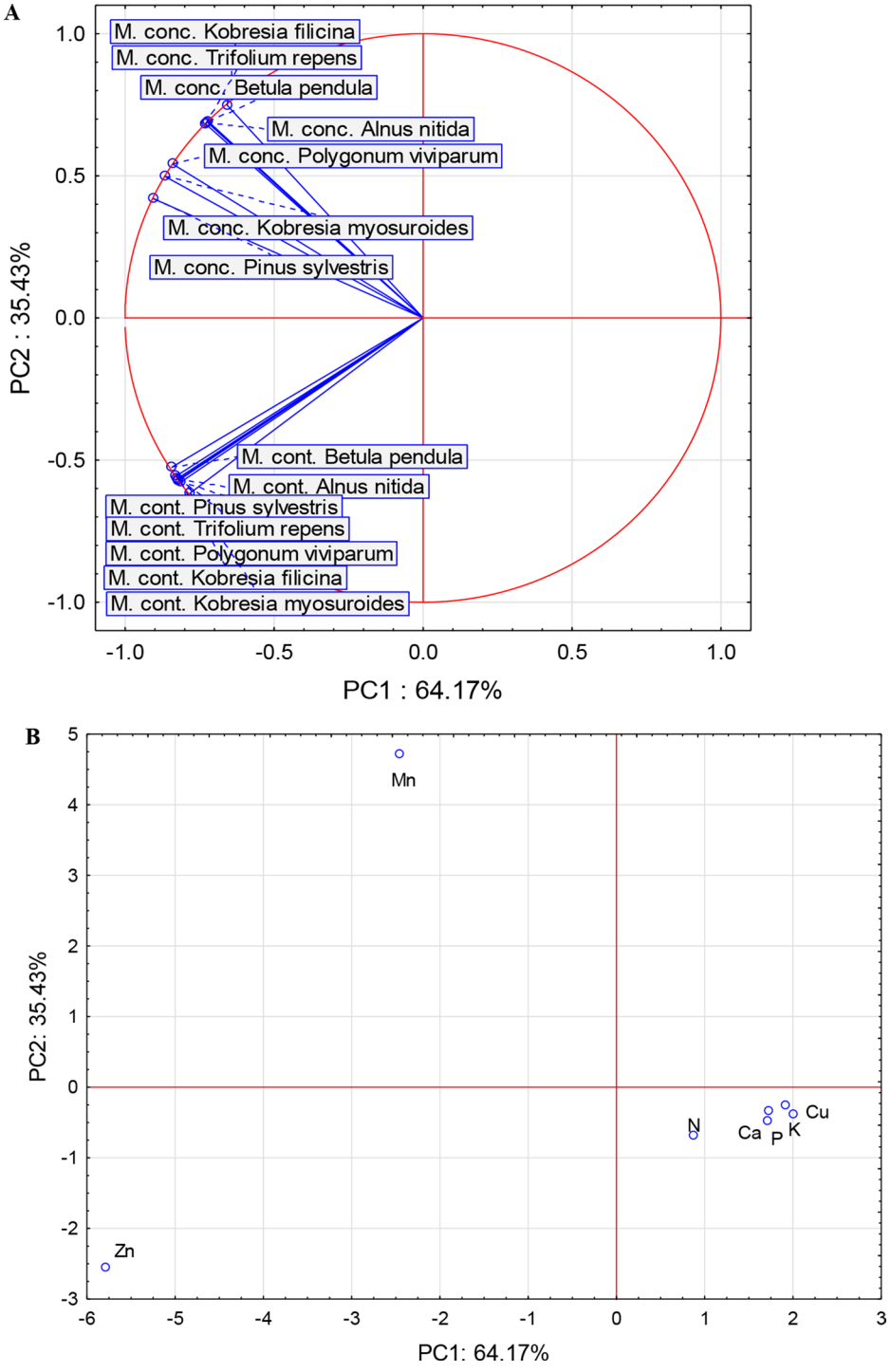

Fig. 4 (**A**) Projection of variables: parameters on the PC1 and PC2 loadings plot, (**B**) Projection of sample type on the PC1 and PC2 scores plot

value, the stronger the linear relationship between the variables. The sign of the correlation coefficient indicated a positive or negative correlation between the studied variables. A heat map is a graphical presentation of the data in which the individual values contained in the matrix are represented by a colour scale. The heat map visualized the data through color differences, which can be seen on the chart as differences, where there were higher values.

Discussion

Kobresia is a small genus of Cyperaceae dominant in the alpine ecosystems, while Polygonaceae ("perennial herbs") are cosmopolitan in northern temperate regions

Table 7 Heat map of the correlation matrix for the tested samples of chemical elements

r>=	-1	-0,80	-0,60	-0,40	-0,20	0	0,20	0,40	0,60	0,80	1

	M. cont. Polygonum viviparum	*M. cont. Kobresia filicina*	*M. cont. Kobresia myosuroides*	*M. cont. Alnus nitida*	*M. cont. Betula pendula*	*M. cont. Pinus sylvestris*	*M. cont. Trifolium repens*
M. conc. Polygonum viviparum	1,000	0,955	0,999	0,980	0,982	0,990	0,982
M. conc. Kobresia filicina	0,955	1,000	0,939	0,994	0,989	0,905	0,993
M. conc. Kobresia myosuroides	0,999	0,939	1,000	0,969	0,970	0,996	0,971
M. conc. Alnus nitida	0,980	0,994	0,969	1,000	0,997	0,944	1,000
M. conc. Betula pendula	0,982	0,989	0,970	0,997	1,000	0,946	0,997
M. conc. Pinus sylvestris	0,990	0,905	0,996	0,944	0,946	1,000	0,947
M. conc. Trifolium repens	0,982	0,993	0,971	1,000	0,997	0,947	1,000
M. cont. Polygonum viviparum	0,386	0,121	0,432	0,209	0,203	0,509	0,216
M. cont. Kobresia filicina	0,368	0,110	0,414	0,195	0,184	0,489	0,202
M. cont. Kobresia myosuroides	0,322	0,061	0,367	0,144	0,137	0,445	0,151
M. cont. Alnus nitida	0,383	0,111	0,430	0,203	0,199	0,507	0,211
M. cont. Betula pendula	0,428	0,152	0,473	0,246	0,249	0,549	0,254
M. cont. Pinus sylvestris	0,380	0,113	0,426	0,201	0,197	0,503	0,209

of the world. "Anjabar or Maslum", the common name of *Polygonum viviparum* and easily grow at mountainous territory of Hindu Raj Mountains [29, 30]. Among the herbaceous plant species, the dicotyledenous herb *P. viviparum* and monocot *Kobresia* have typical ECM short roots with sheaths and Hartig nets. The AM (arbuscular mycorrhizal) fungi colonize neither of these plants. Ascomycete fungi make ectomycorrhizal root tips of the plants [31, 32]. *P. viviparum* and *K. myosuroides* happily associate with ECM fungi [19]. Herbaceous plant growth is severely constrained by the growing season and temperature. As a result, plants create mycotrophic arrangements to maintain the species' fitness and functionality [33].

The sedge family Cyperaceae, which includes *Kobresia*, is frequently found in stress-inducing environments. Numerous studies have identified the ECM fungal communities of *K. bellardii* [32, 33]. According to Gardes and Dahlberg [34], this is one of the earliest ectomycorrhizal plants to generate the thick pads. To preserve their existence in a non-mycorrhizal condition, tussock-forming sedge quickly absorbs the amino acids [34].

Researchers have investigated the anatomical composition of ECM that develops on the roots of *K. bellardii*. These structures, which demonstrated a mutually beneficial relationship between the plant and fungi, displayed a sophisticated arrangement with clear layers of fungal mantle, Hartig net, and aerenchyma [35, 36].

Dauciform roots are found in sedge species, where limited nutrients and adverse environmental conditions forced the species to make mycorrhizal links. In reaction to phosphate deprivation, Cyperaceae plants (*Schoenus unispiculatus* and *Caustis blakei*) produce dauciform roots [37]. The ECM-colonized roots may be the outcome of an ecophysiological response to alpine adversity, where organisms face the environmental stress. Massicotte et al. [36] described the ECM fungal-based anatomical features of *Kobresia* and *P. viviparum*.

The mycorrhizal status of *Polygonum viviparum* is confirmed [36]. This is an Arctic-alpine herbaceous species of high mountainous regions of the northern hemisphere [38]. Due to several limitations, the mountain compelled the plants to develop symbiotic associations [39]. Ectomycorrhizal root tips of *P. viviparum* comprised Hartig net [32], whereas a few authors reported the absence of ECM fungi in their observations [3, 39]. The lack of variation in the ECM of *P. viviparum* has resulted in a paucity of literature on the existence or absence of ectomycorrhizal structures [40]. Olive-green, pseudoparenchymatous angular mantles of *P. viviparum* and black mycorrhizae lead to their relationship with several trees [3, 41]. Thick mantles enclosed the root apex similarly to the ECM of woody dicotyledonous angiosperms [42]. Ectomycorrhizal and arbuscular mycorrhizal fungal colonization was found in the root system of *P. viviparum* [42–44]. Fontana [44, 45] examined the different fungal morphotypes in *P. viviparum.* Additionally, ectomycorrhizae and ectendomycorrhizae have been documented in *P. viviparum* (a dicotyledonous angiosperm) [46, 47].

Neither Bledsoe et al. nor Adams et al. [3, 41] discovered the ECM in *P. viviparum*. A few *Polygonum* species, like Canadian *P. viviparum*, do not have ECM mycorrhizal association but have dematiaceous surface hyphae. The VAM (vesicular-arbuscular mycorrhizal) is also reported in this species [48]. The ectomycorrhizal linkages in the genera *Kobresia* and *Polygonum* were shown in several research studies, similar to this one [16, 18, 19, 35, 46].

El Omari and El Ghachtouli [49] stated that a multitude of fungal species are extensively employed as biocontrol agents in cultivated habitats. The favorable effects of ECM on plant growth are generally acknowledged since it enhances the absorption of water and nutrients, particularly phosphate. Additionally, it provides protection against both biotic and abiotic challenges. Studies of numerous plant species have yielded a wide array of plant responses to colonization. Certain plant species typically do not produce easily identifiable mycorrhizas, most likely because the cost outweighs the benefits. Estimates differ, but research has demonstrated that plants distribute anywhere from 4 to 20% of photo assimilates to mycorrhizal roots. Some species, on the other hand, are facultative mycorrhizal species. Unlike obligatory plants, these species rely on mycorrhizae only when there is a lack of nutrients in the soil.

ECM fungi play a crucial role in forest ecosystems, agriculture, horticulture, biotechnology [50], and environmental sustainability [51]. They have potential applications including biofertilization, biocontrol of plant diseases, and bioremediation of polluted soils [52].

Progress in genomics and molecular biology methods can improve our comprehension of ECM fungal biology, encompassing their genetic variability, evolution, and relationships with host plants and other microbes [53]. ECM fungi contribute to the resilience of forest ecosystems in the face of climate change, helping to reduce its negative effects [54]. The symbiotic relationships between ECM fungus and host plants can optimize the interactions between plants and microbes, leading to improve the functioning of ecosystems [9]. Conducting thorough assessments of ECM fungal diversity and distribution patterns in various biomes and geographical regions will enhance their worldwide biogeography and areas with high biodiversity [55].

Soil is a dynamic biological reactor, where various biochemical reactions and ecological processes occur. The fertility of soil is enhanced by synergistic interactions, competition, and parasitism among microorganisms [56]. Mycorrhiza plays a crucial role in maintaining soil fertility at the global level, addressing significant threats such as soil erosion, soil organic carbon, and nutrient imbalance [57]. Unsustainable land management practices have led to the depletion of soil fertility [58]. ECM has been identified as an eco-friendly approach to improve soil fertility [59]. ECM form a symbiotic relationship that increases the water and nutrient uptake [60].

Mycorrhizal associations can significantly contribute to the decomposition and mineralization of plant organic matter, mobilizing nutrients, particularly nitrogen, for the host plant's benefit. Nitrogen (N) in minerals forms a crucial component of plant growth present in the soil. The plants weakly absorb this, but ECM helps in

mobilization of inorganic nitrogen from the soil [60, 61]. Mycorrhizal roots generate a carbon sink demand, which is amplified by the rise in atmospheric CO_2, hence promoting the growth of ECM. These interactions enhance plant growth, protection, acquiring nutrients, controlling root infections, and plant tolerance to abiotic stressors [62].

Interactions between ECM and other microbes are dependent on various parameters, including the levels of phosphorus and nitrogen in the soil [63]. There is a mutual beneficial link between ECM fungal populations of the soil influenced by nitrogen and phosphorus levels [64]. In this work, the tolerance of fungal species to low soil temperatures (-125 °C) by using a soil mixture with root fragments, mycelium, vegetative mycelium, spores of ECM fungi, and nutrients level were determined. No thresholds of fungal species survival at -125 °C were noted. However, the differences were found in the development and function of mycorrhiza after the exposure at the lowest soil temperature treatments. For frost-tolerant species, the differences as detected in short-term lab treatments reflected the actual differences between the organisms in field conditions too.

The hypothesis was supported by the data regarding the better performance of ECM after very low temperature exposure and clearly observed ECM formation. The ECM supported the plant species *Polygonum viviparum*, *Kobresia filicina*, and *K. myosuroides.* In temperate northern areas, a marked increase in AMF taxa was found in the spring, while the diversity increased in the later growing season. Seasonal changes are attributed to increasing the soil temperature directly or by the availability of carbon from the host [32]. This was remarkable in the study: frost treatments of soil did not influence the proportion of ECM root tips at all. In pure ECM mycelium culture, the growth is even better after exposure to freezing temperatures than unfrozen control, which may be due to minimum competition between the colonies or by activation of mechanisms of physiological recovery after frost [65]. This recovery was favourable for growth and mycorrhizae formation. Similarly, in this experiment during the plant's growth, unlike in nature, the soil temperatures typically remained low in early summer, long after the air temperatures in the growing seasons [66]. Soil temperature of 5 °C in Scots pine resulted in poor root growth, but at 9 °C to 17 °C, ECM formed in all nearby available short roots [67].

Many soluble or exchangeable soil nutrients were found in larger concentrations after the frost exposures. The increased availability was a reflection of foliar nutrients concentration, particularly *K. myosuroides* (Cu, Fe, K, Mn, Zn) [68]. The nitrogen was the only exception, in which both NH_4-N and NO_3-N reduced at all frost treatments. This recommends a change in microbial function by the freezing treatments. In Maljanen et al. [69] study on spruce forests, decreased soil temperature increased both the concentration and emission of soil NO_3 and N_2O, respectively. Here in this study, NO_3 reduced in the frozen treatments due to leaching from the pots, denitrification, or gaseous 'N' losses. Gaseous 'N' loss and denitrification after freezing in soils is a well-known phenomenon [70]. The possible mechanisms of maximum 'N' availability for denitrifying and other microbes from the litter are disrupted by freeze and thaw, and the competition is reduced for the soluble 'N' by other microbes [71–73]. According to Shane et al. [74], this form of root is morpho-physiologically adapted to grow in nutrient-poor soil and facilitates the plants to get nutrients from the stress-inducing soil.

Here we have shown that ECM of *Polygonum viviparum*, *Kobresia filicina*, and *K. myosuroides* survived in a short exposure of lower temperatures. The soil and foliar nutrient concentrations were determined to assess the direct effects of frost on the soil nutrients and thereby plant nutrition versus nutrient effects caused by changes in the root symbioses. However, the leaf 'N' concentration and content decreased with decreasing temperature due to 'P' deficiency.

Conclusion

In conclusion, there was evidence of better performance of ECM colonization ability after the more severe frost treatments. ECM are more tolerant towards freezing temperatures and affect the availability of soil nutrients for different plant species used in this work of cold-stressed areas.

It may not be possible to determine the mycorrhizal capacity of plants growing in a single soil by just looking at their roots. However, soil and environmental factors influence the mycorrhizal fungal associations in a plant species. The pattern of dispersion of roots within the soil profile and the nutritional conditions are both favourable factors for ECM colonization for plant survival in cold areas.

Future prospects

The potential of ECM fungi is a promising tool to address the difficulties in biotechnology, forestry, agriculture, and environmental conservation. The ecological roles of ECM fungus in soil ecosystems, including their involvement in aggregation, breakdown of organic matter, and cycling of nutrients can also be investigated. ECM fungi can be genetically modified using synthetic biological techniques to tailor them for precise agricultural and environmental uses. There is a chance for fungal populations to shift from ECM to ERM (ericoid mycorrhizal) fungal associations in the near future. Further studies are needed on the topic of the after-effects of different soil

temperatures for a longer time and how the following springtime conditions affect the growth and functioning of ECM.

Supplementary Information

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Supplementary Material 1

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Author contributions
AU, LD designed the experiments. AU, FA performed the experiments. AU, MAM, MG analyzed and interpreted the data. AU, MAM, FA, wrote and reviewed the manuscript. MG, FAl commented on the manuscript. FA, AU, MAM reviewed and addressed the revision comments with their expertise. All authors read and approved the final version.

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Data availability
The data set generated and analyzed during the current study are available on special request from the corresponding author.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication
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Competing interests
The authors declare no competing interests.

Author details
[1]Institute of Botany, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan
[2]Department of Botany, Faculty of Science, Fayoum University, Fayoum 63514, Egypt
[3]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia
[4]Department of Chemical Engineering, Qatar University, Doha 2713, Qatar
[5]Laboratoire CHEMBIOPRO (Chimie et Biotechnologie des Produits Naturels), Université de La Réunion, ESIROI Département agroalimentaire, 15 avenue René Cassin, Saint-Denis F-97490, France
[6]Faculty of Production and Power Engineering, University of Agriculture in Krakow, Balicka 116B, Krakow 30 149, Poland
[7]Institute of Agrophysics, Polish Academy of Sciences, Doświadczalna 4, Lublin 20-290, Poland

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