Research Square

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Seedling age is a key determinant of plant adaptability at lower altitudes in the threatened Himalayan medicinal herb Arnebia benthamii (Wall. ex G. Don) Johnston

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Research Article

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Abstract

Himalayan mountains host unique alpine species adapted to extreme conditions. *Arnebia benthamii*, as a characteristic alpine species, constitutes an integral component of the Himalayan Mountain ecosystem. Yet, overexploitation in its native habitat has caused population decline, necessitating conservation through cultivation at lower altitudes. The study evaluated age-dependent transplantation success and post-transplant physiological performance of *A. benthamii* seedlings transferred from high-altitude polyhouse conditions (2646.32 ± 9 m asl) to a low-altitude polyhouse condition (1,530 m amsl). Seedlings aged 1 (1M), 3 (3M), and 6 (6M) months were assessed for survival, growth (rhizome and shoot development), and biochemical parameters (total phenolics, antioxidant activity, and chlorophyll content). Results showed that 6-month-old seedlings had the highest survival rate (83.33%) and significantly ($p < 0.05$) superior growth and biochemical traits as compared to younger age seedlings. Six-month-old seedlings were found optimal for lower-altitude transplantation. By linking seedling age with adaptive responses under altered microclimatic conditions, the study offers a practical strategy for large- scale propagation, cultivation and unique opportunity to increase the economy of local farmers.

Introduction

Mountain system represents complex socio-ecological systems, where human activities and ecological processes are shaped by steep elevational gradients, geological dynamism, and pronounced climatic heterogeneity **(Körner and Ohsawa, 2006; Payne et al., 2020).** Among these, The Himalayan mountains constitute the world's youngest, highest and most dynamic biodiversity hotspots **(Anthal et al., 2026)**, ranging from driest to wettest areas. They host fragile Alpine ecosystem above treeline, characterized by extreme climatic conditions, short growing season, slow regeneration, specialized plant adaptation, and shallow nutrient poor soils **(Korner, 2023; Williams et al., 2014)**. Elevational gradients strongly influence plant growth patterns and stress-adaptation strategies **(Khatoon et al., 2025)**. As a characteristic alpine species, *Arnebia benthamii* forms an integral component of the Himalayan Mountain ecosystem. However, it faces significant threats, with its population experiencing a steep decline. It was reported as endangered in Jammu and Kashmir in 2003 **(Baig et al., 2014)** and currently listed as 'Data Deficient' (DD) on the IUCN Red List 2024 (https://www.iucnredlist.org/species/216172075/216202474**).**

Arnebia benthamii (Wall. ex G. Don) Johnston is a high-value perennial, medicinal plant belonging to Boraginaceae, commonly known as Ratanjot **(Ganie et al., 2014)**. The species is valued for its rhizome, which contain shikonin (napthoquinone), a red pigment widely used for various pharmaceutical properties. Owing to its medicinal significance, *A. benthamii* contributes substantially to traditional Unani-Ayurvedic pharmacopeias and commercial formulations like Gaozaban, while supporting rural livelihoods for promoting hair growth, curing cough, cold and fevers. The estimated annual trade of *A. benthamii* in India is 100–200 MT **(NMPB, 2022).** Ecologically, *A. benthamii* is adapted to well- drained, coarse-textured alpine soils and survives under nutrient limited conditions **(Ganie et al., 2014**). Despite its significant medicinal and economic importance, its natural habitat alone is not sufficient to sustainably supply the raw material required for various uses.

Increasing commercial demand for alpine medicinal plant has intensified harvesting pressure across the Himalaya, often exceeding the regenerative capacity of fragile mountain ecosystems. In the case of *A. benthamii*, premature collection of rhizomes much before flowering, which not only prevents natural seed production and regeneration but also responsible for the declining population of the species in the natural habitat **(Manjhkola and Dhar, 2002)**. Moreover, the grazing by animals, particularly targeting the flowering shoots and leaves **(Ganaie et al., 2010)**, has further hindered its natural population recovery. These pressures have resulted in marked population declines across parts of its distribution range, highlighting a growing mismatch between medicinal plant demand and the ecological limits of the alpine system. This supply–conservation conflict underscores the urgency of developing sustainable cultivation and conservation strategies for *A. benthamii*.

In mountain ecosystems, direct seeding is often constrained by poor germination rates, uneven seedling emergence, susceptibility to environmental stresses, competition from weeds and vulnerability to diseases **(Palma et al., 2015)**, including fluctuations in temperature and moisture. These factors can lead to high seedling mortality and lower overall plant survival. As a result, transplanting is a critical step in the cultivation of medicinal plants, as it ensures that only healthy and vigorous seedlings are established in the main field. This process allows for the selection of uniform, disease-free, and well-developed plants for transplanting, thereby improving the chances of successful crop establishment and optimal yield. Nevertheless, the careful selection of seedling age is crucial in ensuring uniformity of growth and development, leading to more consistent and reliable phytochemical profiles **(Bansal and Priyadasini, 2021)**. Therefore, transplanting seedlings emerges as a critical intervention strategy. However, the success of such restoration efforts is intricately linked to the complex interplay of microclimatic environmental factors that can substantially influence plant survival and establishment **(Butola et al., 2009).** In this context, besides open field conditions at different altitudes, protected cultivation environment such as glasshouse, polyhouse, greenhouse, shade net etc. plays a critical role in medicinal plant propagation and conservation. Under such controlled conditions, key growth factors like temperature, relative humidity can be regulated to create optimal conditions for plant development, ensuring robust and healthy plant growth. Precisely regulating growth parameters under protected cultivation ensures shielding of medicinal plants from external threats like animal grazing, which subsequently maximises the accumulation of bioactive compounds, ensuring product quality, and improved plant secondary metabolite production **(Stutte, 2016)**. Moreover, it also provides a transitional buffer, enabling high-altitude alpine plants to adapt to the challenges of low-altitude conditions and thereby strengthening the effectiveness of ex situ conservation, restoration planning, and sustainable cultivation strategies in Himalayan landscapes.

The previous studies have demonstrated successful domestication of the high-altitude inhabiting plant species, *A. benthamii* (3000 to 3900 m amsl), at lower altitudes (1450 m amsl) (**Ganaie et al., 2010). Manjhkola and Dhar (2002)** reported 67% and 60% seedling survival rate of *A. benthamii* at sites other than the native habitat sites *viz*., Lata (2220 m amsl) and Katarmal (1240 m amsl), respectively. They have focussed on site suitability and overall survival of the plant and recommended that establishing

nurseries at lower altitudes or propagating seedlings under controlled conditions could serve as an effective strategy for providing propagation materials to inhabitants of high-altitude villages.

Despite successful domestication demonstrations, no studies have examined age- dependent transplantation success of *A. benthamii* at lower altitudes. The present study addresses this critical gap by evaluating optimal age of transplanting the seedlings of *A. benthamii*, survival % and growth performance of different-aged seedlings after transplantation at a lower altitude-controlled polyhouse condition. Physiological and biochemical parameters were assessed to characterise the overall health and adaptability of different age plants post-transplantation. By linking seedling age with adaptive responses under altered microclimatic conditions, the study provides a practical framework for large scale propagation, ex-situ conservation, and sustainable domestication of this endangered medicinal plant.

Materials and Methods

1.1. Site description and experimental details

Seeds of *A. benthamii* were collected in October, 2022 from Balpata bugyal (30°12'22"N, 79°39'47"E, 3,354 m) in Chamoli district, Uttarakhand. The plant's herbarium voucher, accession number 1567, is preserved in the Department of Biological Sciences Herbarium (GBPUH). The plant was identified by Dr. D.S. Rawat. The seeds were sown directly in black low- density polyethylene (LDPE) nursery bags (size: 8” x 10” thickness: 200 gauge) for germination on 4th April, 4th July and 4th September, 2023, under controlled polyhouse conditions maintained at the day/night temperature range of 21–25 ^{0}C/ 14–18 ^{0}C with relative humidity (RH) of 60–80% in village Pader in Chamoli (30°18'35.87"N, 79°31'58.57"E, 2646.32 ± 9 m asl) Uttarakhand. The bags were perforated to ensure adequate drainage and aeration. Plants were transplanted on 4th October, 2023 at Padwadangar under polyhouse conditions (1,530 m above mean sea level; latitude 29.33°N, longitude 79.43°E), maintained at day/night temperature range of 21–25 ^{0}C/ 14–18 ^{0}C with relative humidity (RH) of 60–80%. For transplantation, plastic pots (2 Kg capacity) measuring 9.75 inches in diameter and 9.12 inches in height were filled with a sand-to-soil mixture in a 2:1 ratio. Seedlings were divided as per their age groups *viz.* one month old (1M), three months old (3M) and six months old (6M), respectively. Thirty seedlings from each age group were transplanted from the LDPE nursery bags into the pots, ensuring that the original root structure remained undisturbed. Adequate soil moisture was maintained by regularly watering the soil as per standard recommendations.

Observations

2.2. Survival percentage

The success rate of transplantation was assessed on the 30th day after transplantation by calculating the percent survival (%), which was determined by dividing the number of surviving seedlings by the total number of seedlings planted multiplied by hundred.

2.3. Growth and biochemical traits

Three biological replicates were taken from each age group in completely randomized order to analyze the growth and biochemical parameters. The growth parameters, such as the number of newly emerged leaves per plant, shoot length, root length and width of main root, were evaluated for 1-month (1M), 3-month (3M) and 6-month-old (6M) plants, respectively. The length of each rootstock was measured from the root-shoot junction to the root tip. The root width was measured from the thickest part of the rootstock with the help of a vernier caliper. The root length and diameter were evaluated at two timepoints *viz.* before transplantation (T0) and six months post-transplantation (T6M) to assess the growth. Conversely, shoot length and the number of leaves per plant were measured only for the plants matured after transplantation.

2.4. Biochemical parameters

Due to the poor survival of 1M plants, the biochemical parameters were evaluated for plants transplanted at the age of 3-month-old (3M) and 6-month-old (6M) plants only after six months from transplantation.

2.4.1. Chlorophyll and carotenoid content

Total chlorophyll and carotenoid content was estimated as per **Hiscox and Israelsham (1979)**. The fresh leaves (50 mg) were suspended in 10 mL Dimethyl Sulfoxide after cutting into small pieces and kept in a water bath for 3h at 60^0C. The mixture was maintained at room temperature for 1h, decanted, and the absorbance of the leaf extract was taken at 663 and 645 nm against a DMSO blank. The carotenoid content was estimated using the same leaf extract by measuring the absorbance at 480 nm. All the quantifications were carried out in triplicate.

2.4.2. Total phenol content and antioxidants

The leaf samples of *A. benthamii* were washed with tap water twice. In order to facilitate grinding and minimize degradation of bioactive constituents, 0.5 gram of leaf samples were homogenized in a chilled mortar-pestle in methanol. The suspension was centrifuged at 10,000 x g at 4 ^{0}C for 30 min. The supernatant formed crude methanolic extract and then measurement of phenol content **(Singleton & Rossi, 1965)** and total antioxidant activity **(Vongsak et al., 2013)** were performed with minor modifications.

0.5 mL of extract was combined with 0.2 mL of Folin-Ciocalteu reagent and Sodium carbonate (7%) was added to the mixture after 5 min. Absorbance of the reaction mixture (blue-coloured) was measured at 765 nm using spectrophotometer (Genesys, Thermo-Fisher). The measurement was referenced against a standard curve prepared from gallic acid solutions, and results are presented as milligrams of gallic

acid equivalents (mg GAE) per gram of fresh weight. DPPH (2, 2'-diphenyl-1-picrylhydrazyl) radical scavenging activity (%) was assessed following **Vongsak et al. (2013)**. DPPH solution (3mL, 0.1 mM) was mixed with 1mL plant extract (100 µg/mL) or solvent (control). The reaction mixture was incubated in the dark for 30 min. The absorbance was detected at 517 nm using spectrophotometer (Genesys, Thermo-Fisher). The following formula was used to determine the scavenging activity.

$$\text{Scavenging Potential (\%)} = \frac{(ac - as)}{ac} \times 100$$

where, ac = the absorbance of the control sample and as = the absorbance of the plant sample.

2.4.3. Total superoxide dismutase (SOD) activity

An amount of 0.2 g fresh leaf sample was extracted with 2 ml of cold extraction buffer comprising 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM sodium ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP). The homogenate was subjected to centrifugation at 10,000 rpm for 20 min at 4^0C. The supernatant obtained after centrifugation was directly used for the enzyme assay. SOD activity was determined by measuring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) **(Giannopolitis and Ries 1977)**. The reaction mixture, with a total volume of 3-ml, comprised 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 2 µM riboflavin, 0.1 mM EDTA and 100 µl enzyme extract. The reaction mixtures were exposed to illumination at 5000 lux for a duration of 25 min. The amount of enzyme required to cause 50% inhibition of NBT reduction per unit time, as monitored at 560 nm, is defined as one unit of SOD activity per mg of protein.

2.4.4. Total Protein Content

Total soluble protein was determined as per **Bradford assay (1976)** using Bovine serum albumin (BSA) as standard and was expressed as µg of protein per gram of fresh weight (FW).

Statistical analysis

All the experiments were done in completely randomized design. One-way ANOVA was performed to estimate the effect of plant age on growth and biochemical parameters. Data was analyzed using Genstat 15Ed (VSN International, UK) and the graphical representation of the data were carried out using Sigmaplot 15.0 version (Grafiti LLC, USA). Comparison of mean was performed using Fisher's least significant difference (LSD) test at $P < 0.05$.

Results

3.1. Survival of seedling increased with age of seedling

Transplantation success, evaluated 30 days after transplanting plants from high altitude (2646.32 ± 9 m asl) to low altitude (1,530 m amsl) polyhouse-controlled conditions, revealed significant age-dependent differences in survival rate. The 6M plants demonstrated significantly ($p < 0.05$) higher survival rate of 83.33% as compared to 3M (60%) and 1M plants (6.67%) (Fig. 1). The 1M plants exhibited a very poor survival percentage and did not survive beyond two months, hence, this age group was not considered for subsequent analysis.

3.2. Seedlings with higher age at transplanting showed superior growth and biochemical traits:

A significant increase in rhizome length and width was observed over the six months. The rhizome length of 3M and 6M plants increased significantly by 0.57 cm ($p < 0.05$) and 0.9 cm ($p < 0.05$), respectively (Fig. 2, A). Similarly, the rhizome width of 3M and 6M plants increased significantly by 0.66 cm ($p < 0.05$) and 0.9 cm ($p < 0.05$), respectively (Fig. 2, B). At the time of transplantation, shoot length was 9.23 cm in 3M plants and 9.83 cm in 6M plants. The difference was statistically significant (s.e.d = 0.124, $p < 0.05$) (Fig. 3, A). The number of leaves in 3M plants was 7.33 and for 6M plants was 7.67. The difference was not found statistically significant (s.e.d.=0.471, $p > 0.05$) (Fig. 3, B). Among various photosynthetic pigments, 6M plants exhibit higher significant increase ($p < 0.001$) in chlorophyll b (Fig. 4, B) and moderate increase ($p < 0.05$) in total chlorophyll (Fig. 4, C) and carotenoid content (Fig. 4, D) than 3M plants.

3.3. Phytochemical profiling

In our study, methanolic extract of fresh 3M leaves of *A. benthamii* at a concentration of 100 µg/ml exhibited 81.46% free radical scavenging activity, while the extract from fresh 6M leaves showed a scavenging activity of 88.83%. The difference in scavenging activity between the two age groups was statistically significant (s.e.d.= 0.299, $p < 0.001$) (Fig. 5, A). A significant difference ($p < 0.05$) was also observed in the total phenolic content between the 3M and 6M leaves (Fig. 5, B).

3.4. Total Protein content and Superoxide dismutase activity

The total protein content and SOD activity in 3M plants were recorded as 12.56 mg/g FW and 1.548 Unit/mg FW, respectively (Fig. 5, C), while in 6M plants, it was 14.52 mg/g FW and 1.525 Unit/mg FW respectively (Fig. 5, D).

Discussion

The present study highlights the age-dependent variation in transplantation success, growth performance, and biochemical responses of *A. benthamii* when transplanted from high-altitude polyhouse-controlled conditions to low-altitude, polyhouse-controlled conditions. Our findings clearly

demonstrated that plant age plays a pivotal role in determining post-transplant survival, morphological development, photosynthetic pigments, and antioxidant potential. All these factors eventually determine successful plant establishment. Indeed, transplantation success varied significantly across different age groups, with older seedlings exhibiting better adaptability. Notably, 6-month-old plants showed the highest survival rates, plausibly due to their well-developed root systems, which could facilitate more efficient water and nutrient uptake and mitigated transplant shock. On the contrary, the low survival percentage in 1-month-old seedlings underscored their vulnerability to abrupt environmental shifts, such as changes in altitude and microclimatic conditions. Our observations align with previous studies, such as **Butola (2009)**, who reported superior transplant performance in 90-day-old *Angelica glauca* seedlings compared to younger age groups, attributing their success to enhanced physiological maturity and environmental resilience. The present study is also in line with **Rafique (2023)**, who associated increased plant age with improved water uptake, nutrient assimilation, and antioxidant defense mechanisms. Indeed, a stronger antioxidant defense systems in 6-month-old plants could mitigate oxidative damage induced by transplantation shock and environmental stress due to change in altitude. The significantly higher shoot length in 6-month-old seedlings indicates better resource allocation to aboveground biomass following transplantation. Interestingly, 6-month-old seedlings grew taller as compared to 3-month-old plants, but the rate of leaf production remains relatively consistent during early post-transplant establishment across both the age groups. Conversely, better rhizome length and width in 6-month-old plants further reinforces their successful establishment and adaptation. Seedling morphological traits, especially rhizome and shoot attributes, have long been considered reliable predictors of post-transplant growth and survival **(Puttonen, 1997)**.

The chlorophyll and carotenoid contents serve as reliable indicators of physiological performance and stress response **(Pavlovic et al., 2014)**. Present study also employed DPPH radical scavenging assay to assess antioxidant capacity. The results were comparable to earlier findings from fresh callus cultures of *A. benthamii*, which showed 77–79% scavenging activity under different conditions **(Kumar et al., 2023)**. Other studies have confirmed significant radical scavenging activity in various *A. benthamii* extracts, highlighting its potential as a source of natural antioxidants **(Ganie et al., 2014; Shameem et al., 2015; Parray et al., 2015)**. High antioxidant capacity reflects the activation of plant defense mechanisms to neutralize reactive oxygen species (ROS), thus preserving cellular integrity. The presence of phenolic compounds further supports this adaptation strategy, as they play critical roles not only in stress resistance but also in growth and development **(Hashim et al., 2020; Pratyusha, 2022)**. Biochemical markers such as total protein content and superoxide dismutase (SOD) activity further elucidate the physiological adaptation of transplanted seedlings. Elevated protein levels also indicate active metabolism and structural development of the seedlings. SOD, a key antioxidant enzyme, protects plants from oxidative damage by converting superoxide radicals into less harmful molecules **(Gill & Tuteja, 2010)**. In this study, enhanced SOD activity in 6-month-old plants underscores their superior stress adaptation and resilience potential under changing environmental conditions. The present study, though conducted under controlled polyhouse conditions, provides insights that form a strong foundation for developing field-based cultivation strategies at lower altitudes. However, polyhouse buffering of ambient

conditions limits direct inference to open field conditions. To further expand the applicability of our findings, future research may explore the responses of *A. benthamii* seedlings to transplantation under natural open-field conditions across varying altitudes.

Conclusion

This study demonstrates that seedling age significantly influences transplantation success and physiological performance in *A. benthamii* under low-altitude, polyhouse-controlled conditions. Among the tested age groups, 6-month-old seedlings exhibited the highest survival rate, superior growth and biochemical traits, including increased rhizome development, shoot length, chlorophyll and phenolic content, antioxidant activity, and SOD levels. These results suggest that 6-month-old seedlings are best suited for transplantation and sustainable cultivation of this endangered high-altitude medicinal species. Moving forward, propagation and conservation efforts should prioritize the use of older seedlings (≥ 6 months) to maximize establishment success and long-term viability, especially in restoration and cultivation programs aimed at ensuring the species' survival under changing environmental conditions.

Declarations

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Author contributions

Aabha Bahuguna carried out the experimental work and collected all the data. Experiment was planned by Preeti Chaturvedi, PI of the research project on *Arnebia benthamii.* Aabha Bahuguna, Sugandha Pant and Ishita Chandra processed the data and did the statistical analyses. All authors read and approved the final manuscript.

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Data availability

All datasets generated and analysed during the current study are used and presented in the manuscript and can be made available from the corresponding author on reasonable request.

Ethics approval

This study involved plant material of *Arnebia benthamii* (Wall. ex G. Don) I.M. Johnst., a Himalayan medicinal species categorized as Data Deficient by the IUCN Red List, though considered regionally threatened in parts of its natural distribution. Plant material (seeds) was collected in accordance with relevant institutional, national, and regional guidelines for biodiversity conservation. Necessary permissions for seed collection were obtained from the Regional Forest authority, district Chamoli, Uttarakhand, India. The collected seed was subsequently raised under controlled polyhouse nursery conditions in Village Pader. Permission for plant establishment and experimental use of the plant material was obtained from the Herbal Research and Development Institute (HRDI), Mandal, Gopeshwar, Uttarakhand, India.

Consent to participate

Not applicable

Consent to publish

All authors have read and approved this final manuscript and consent to its publication.

Competing Interest

The authors declare no competing interests.

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Figures

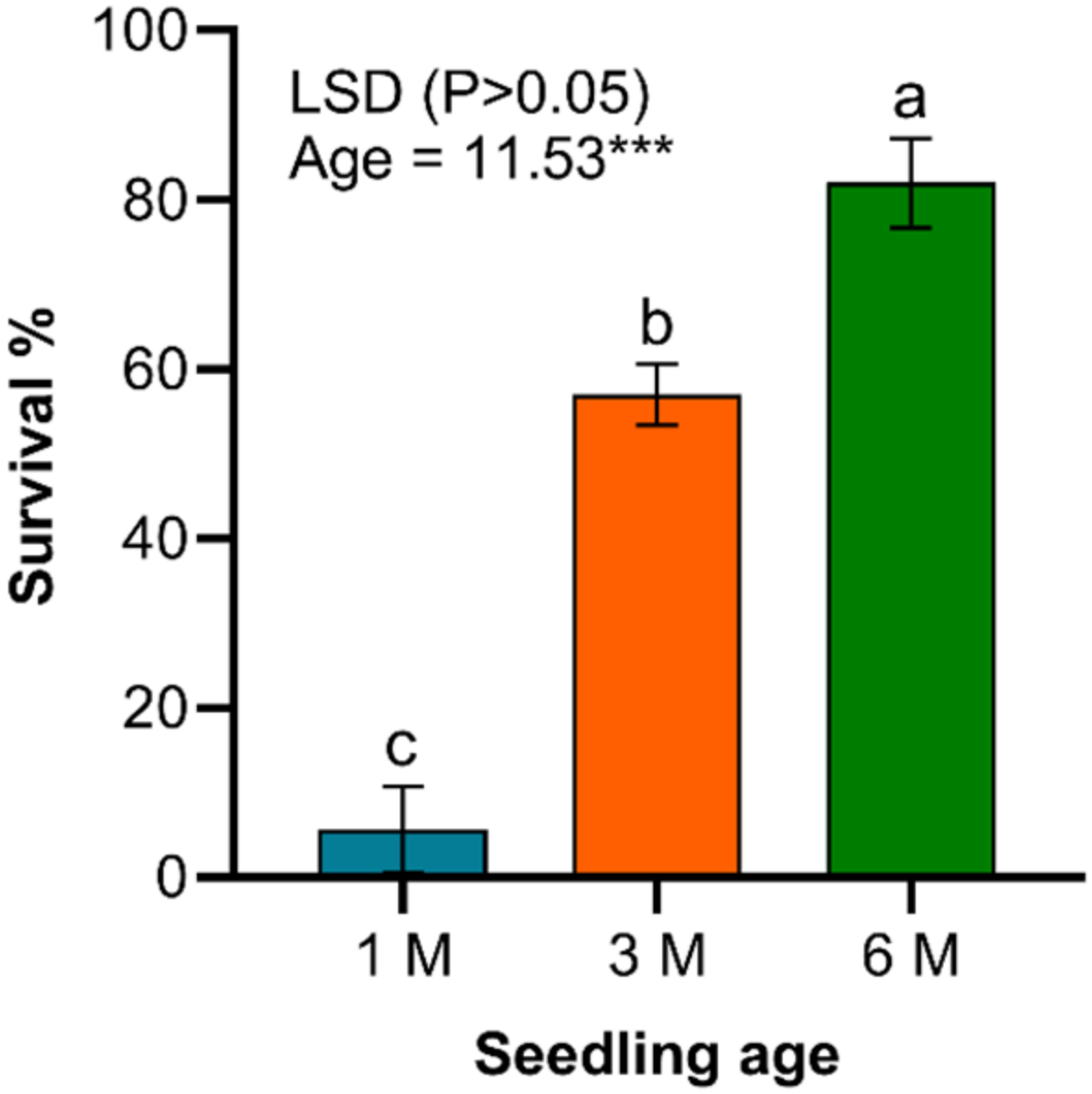

Figure 1

Effect of seedling age at transplanting on plant survival percentage.

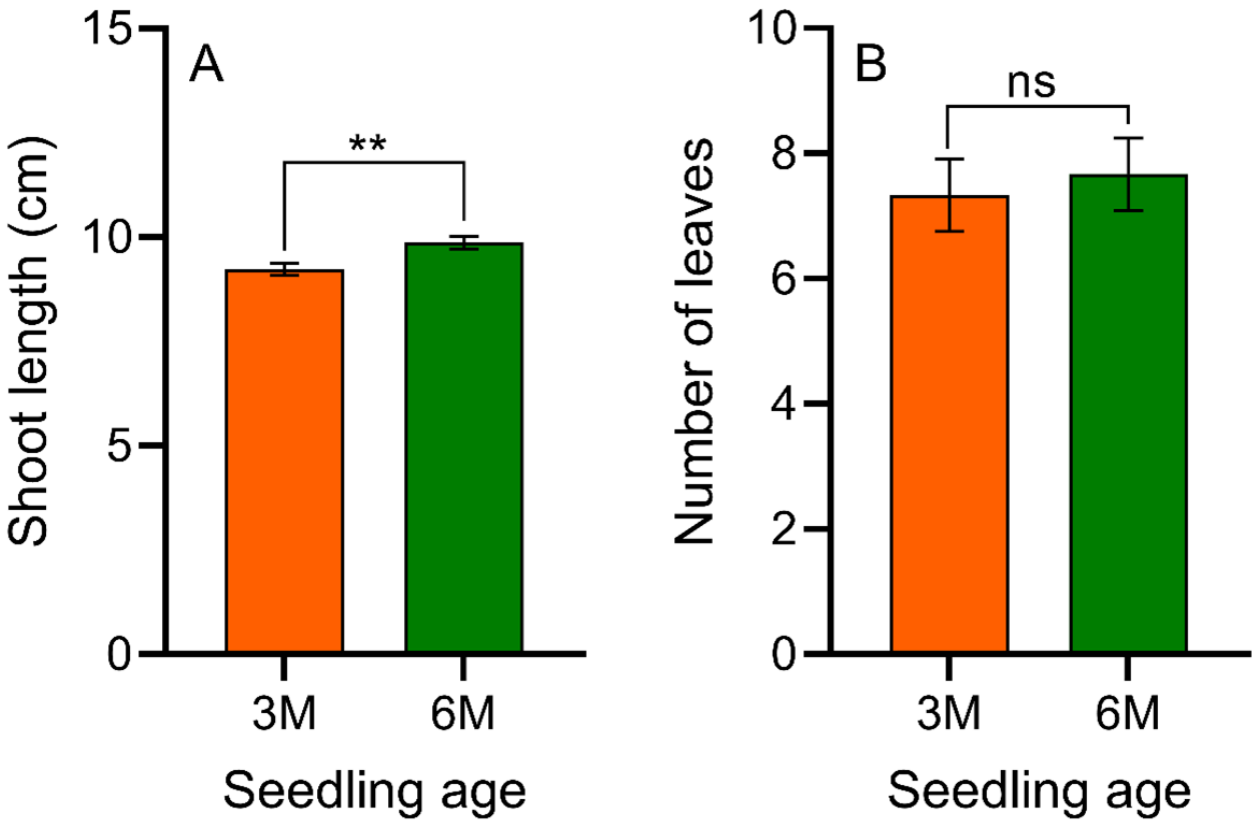

Figure 2

Effect of seedling age at transplanting on shoot and leaf traits: (A) shoot length (cm); (B) number of leaves per plant

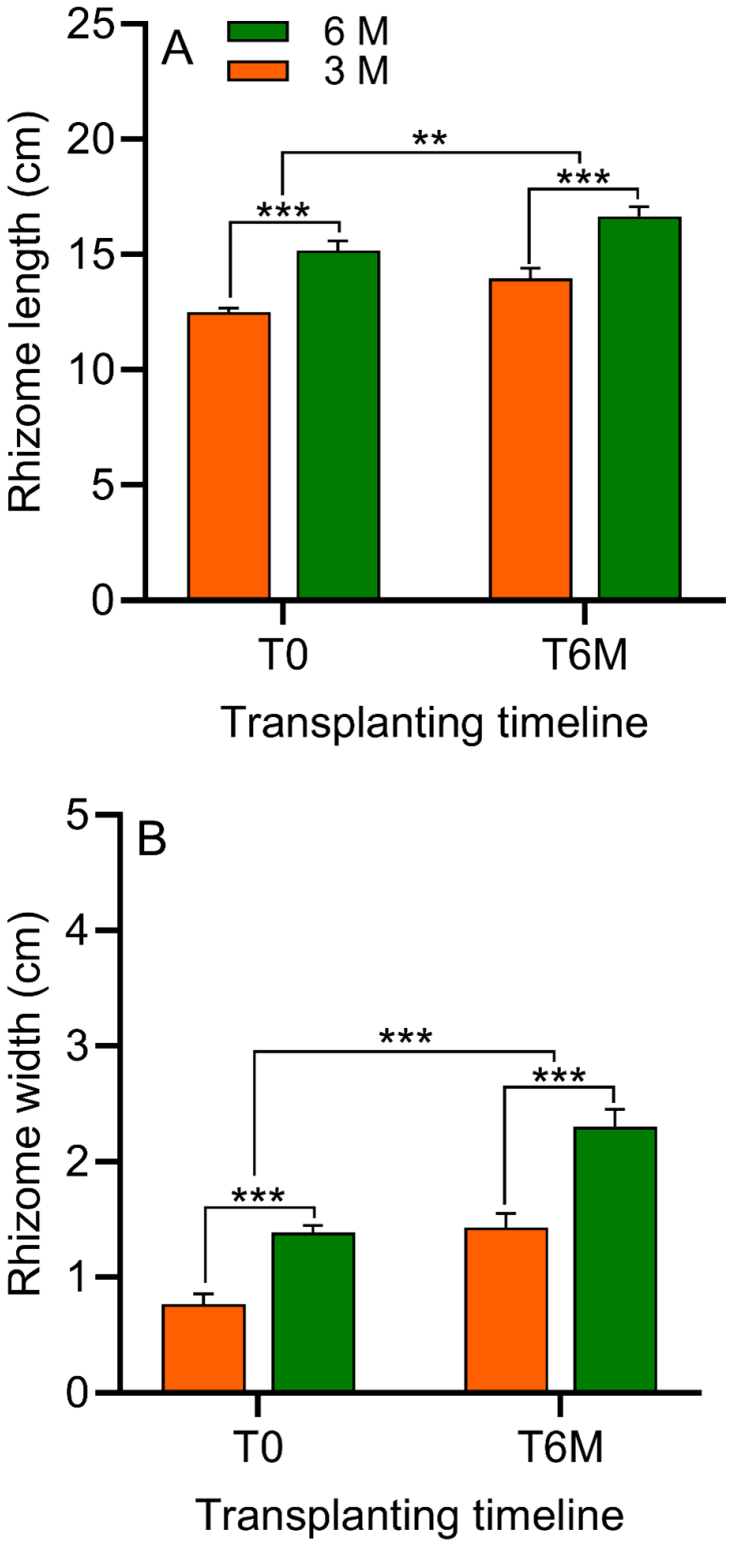

Figure 3

Effect of seedling age on rhizome traits at transplanting (T0) and six months after transplanting (T6M): (a) Rhizome length (cm); (b) Rhizome length (cm)

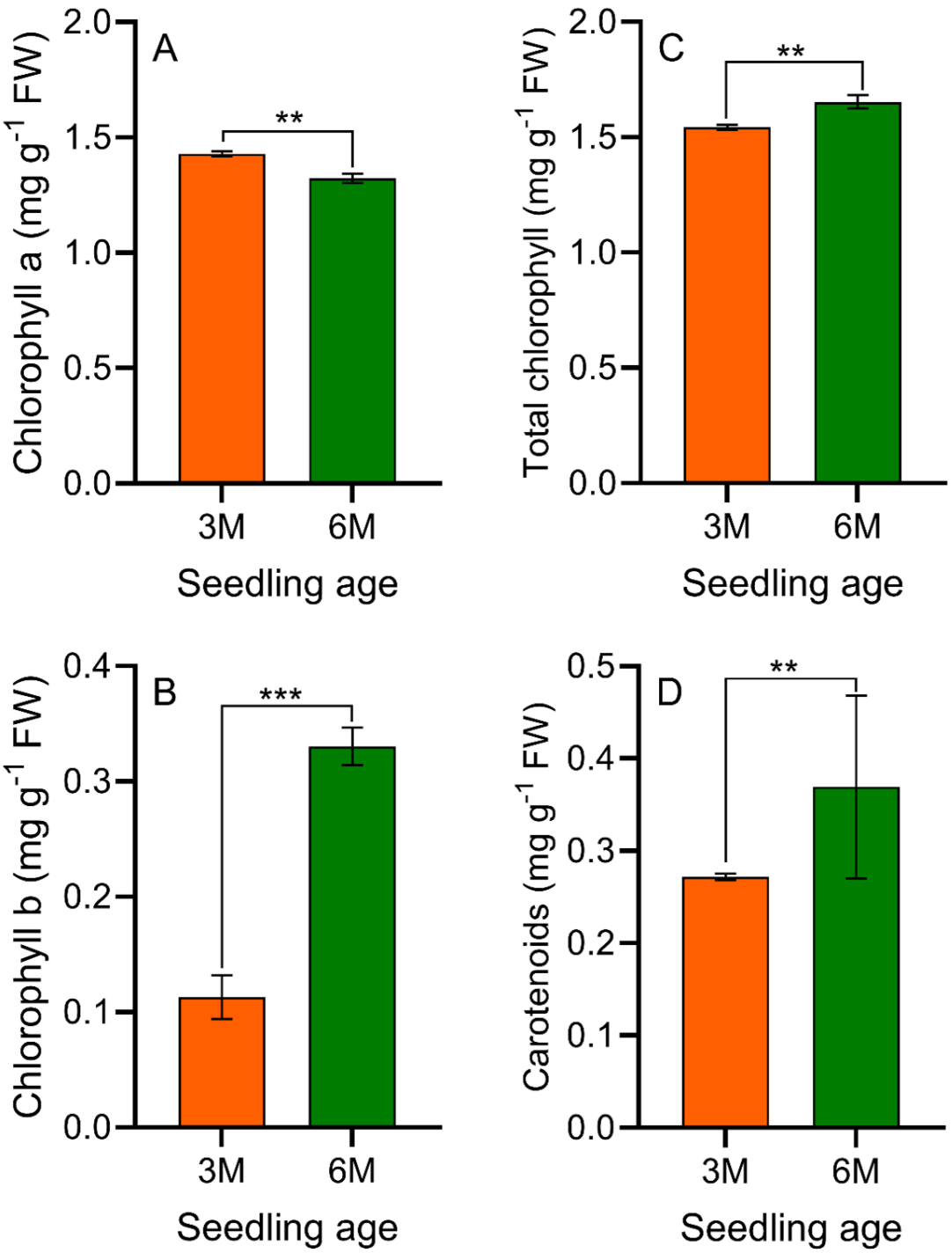

Figure 4

Comparison of chlorophyll and carotenoid content in 3-months and 6-months-old seedlings, (A) Chlorophyll a (mg g^{-1} FW); (B) Chlorophyll b (mg g^{-1} FW); (C) Total Chlorophyll (mg g^{-1} FW); (D) Carotenoids (mg g^{-1} FW)

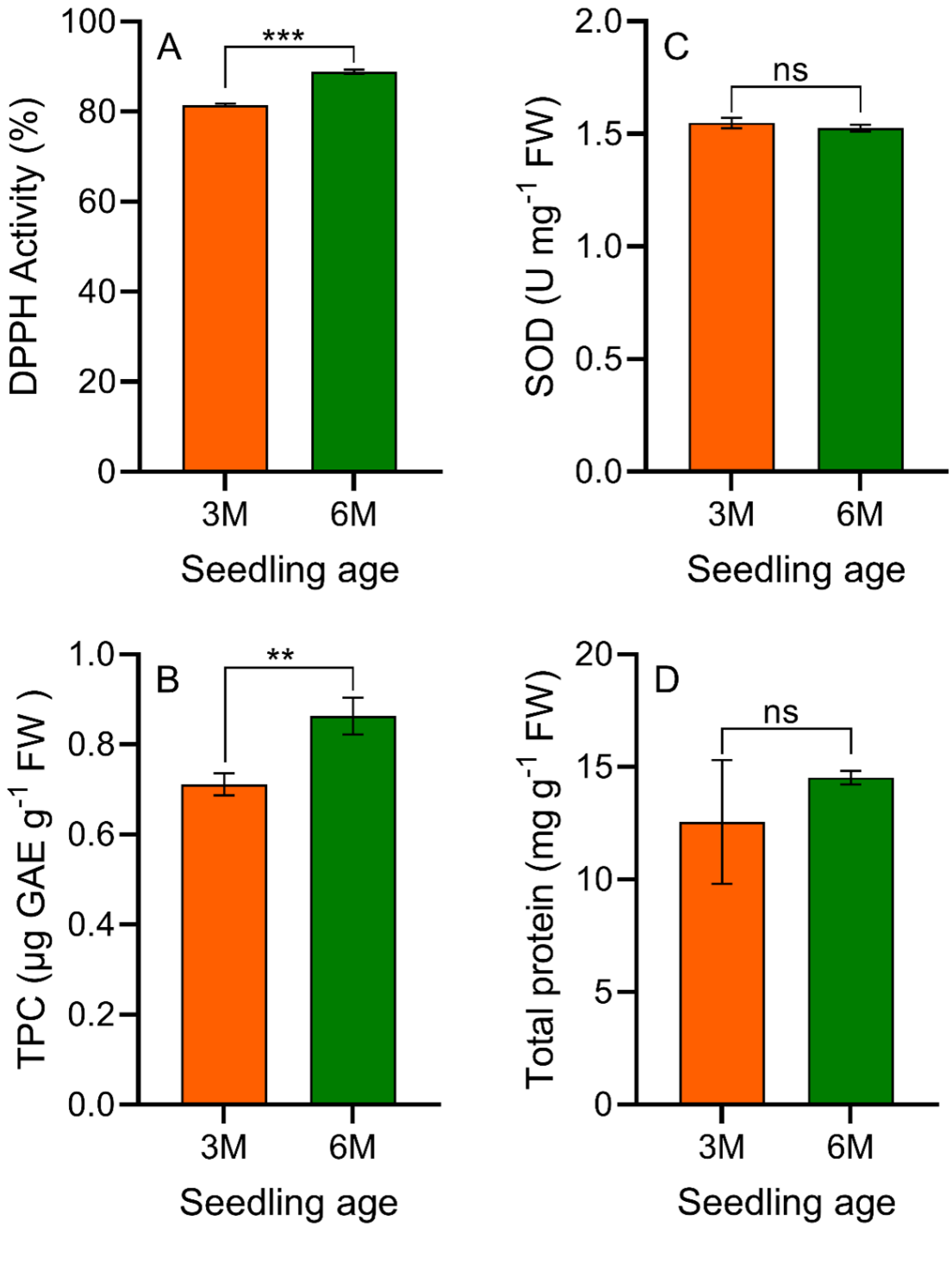

Figure 5

Comparison of antioxidant activity, total phenolic content, superoxide dismutase activity, and total protein in 3 Months and 6 Months old seedlings. (A) 2,2-Diphenyl-1-picrylhydrazyl activity (%); (B) TPC (Total Phenolic Content) (µg Gallic Acid Equivalents g^{-1} Fresh Weight)); (C) Superoxide Dismutase activity (U mg^{-1} FW); (D) Total Protein (mg g^{-1} FW)