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ARTICLE

Quantitative exploration of arbuscular mycorrhiza colonization in *Dactylorhiza hatagirea* (D.Don) Soó in Kashmir Himalaya

Exploração quantitativa da colonização de micorrizas arbusculares em *Dactylorhiza hatagirea* (D.Don) Soó na Caxemira Himalaia

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Abstract: Mycorrhizae, exclusively associated with orchids, play a crucial role in plant growth, competitive interactions and pathogen protection. This study aimed to quantitatively assess the arbuscular mycorrhizal fungi (AMF) colonization rate in *Dactylorhiza hatagirea* at three sites in Kashmir Himalaya: Gulmarg, Sonamarg, and Pahalgam. Additionally, it examines soil parameters such as temperature, precipitation, pH, organic carbon, phosphorus, potassium and nitrogen. The root tips were collected at a depth of 0-20 cm. Samples were stored in sterilized polythene bags, refrigerated at 4±1 °C. Our results showed a significant variation in mycorrhizal colonization across three sites, with Pahalgam showing the highest at 78%, followed by Gulmarg at 73%, and Sonamarg at 57%. Principal Component Analysis (PCA) revealed 69.5% of the variation in environmental and soil variables. Positive correlations were observed among pH, nitrogen, potassium, and organic carbon, whereas phosphorus was negatively correlated with potassium and nitrogen. Gulmarg and Pahalgam were similar in soil characteristics, while Sonamarg exhibited distinct differences. Phosphorus positively correlated with Sonamarg but negatively with Pahalgam and Gulmarg, while other soil parameters showed opposite trends. The study emphasizes the significance of AMF colonization in understanding and enhancing plant-microbe interactions in terrestrial ecosystems, highlighting its role in nutrient cycling and the assimilation of essential elements like phosphorus and nitrogen.
Keywords: arbuscular mycorrhizal fungi, nitrogen, nutrient cycling, orchid himalaia, phosphorus.

Resumo: As micorrizas, associadas exclusivamente às orquídeas, desempenham um papel crucial no crescimento das plantas, nas interações competitivas e na proteção contra patógenos. Este estudo teve como objetivo avaliar quantitativamente a taxa de colonização de fungos micorrízicos arbusculares (FMA) em *Dactylorhiza hatagirea* em três locais localizados na região de Caxemira (Himalaia) : Gulmarg, Sonamarg e Pahalgam. Além disso, examinar parâmetros do solo como temperatura, precipitação, pH, carbono orgânico, fósforo, potássio e nitrogênio. As pontas das raízes foram coletadas a uma profundidade de 0-20 cm e após armazenadas em sacos de polietileno estéreis, refrigeradas a 4±1 °C. Nossos resultados mostraram uma variação significativa na colonização micorrízica nos três locais, sendo que Pahalgam apresentou o valor mais alto com 78%, seguido por Gulmarg com 73% e Sonamarg com 57%. A Análise de Componentes Principais (ACP) revelou 69,5% de variação nas variáveis ambientais e de solo. Correlações positivas foram observadas entre pH, nitrogênio, potássio e carbono orgânico, enquanto o fósforo foi negativamente correlacionado com potássio e nitrogênio. Os locais Gulmarg e Pahalgam foram semelhantes nas características do solo, enquanto Sonamarg exibiu diferenças significativas. O fósforo correlacionou-se positivamente com Sonamarg, mas negativamente com Pahalgam e Gulmarg, enquanto outros parâmetros do solo mostraram tendências opostas. O estudo destaca a importância da colonização de FMA na compreensão e melhoria das interações planta-micróbio em ecossistemas terrestres, destacando o seu papel na ciclagem de nutrientes e na assimilação de elementos essenciais como o fósforo e nitrogênio.
Palavras-chave: fungos micorrízicos arbusculares, nitrogênio, ciclagem de nutrientes, orquídea himalaia, fósforo

Introduction

Arbuscular mycorrhizal fungi (AMF) establish symbiotic associations with the roots of nearly all terrestrial plants, significantly enhancing growth and productivity, particularly under adverse environmental conditions (Khaliq et al., 2022). AMF positively influences plant development by optimizing the acquisition of essential nutrients, including phosphorus, water, and minerals (Siddiqui et al., 2008). The symbiotic interface of arbuscular mycorrhizae facilitates the reciprocal exchange of nutrients, signalling molecules, and protective compounds between the fungal and plant partners. AMF plays a pivotal role in improving plant tolerance and resilience to various abiotic stressors such as drought, salinity, and heavy metal toxicity (RezáCová et al., 2011). These benefits arise from the modulation of antioxidant Défense mechanisms, osmotic adjustment, and hormone regulation in plants colonized by AMF. These adaptive responses contribute to enhanced plant performance, increased photosynthetic efficiency, and elevated biomass production in challenging environmental conditions (Li et al., 2022). The positive impact of AMF extends beyond individual plants to influence soil structure, nutrient cycling, and carbon sequestration, thereby contributing to the maintenance of resilient ecosystems (Mbodj et al., 2018). The effects of AMFs on plant growth and ecological stability exhibit species and environment-specific characteristics. AMF strengthens the plant's immune system, giving it a more extraordinary ability to resist pests and pathogens more effectively (Begum et al., 2019).

AMF initiates morphological, physiological, and molecular responses in plants facing abiotic stress, leading to improved water and nutrient acquisition, enhanced plant development, and increased abiotic stress tolerance. Additionally, AMF-mediated effects are potentiated by essential oils (EOs), superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (A PX), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and phosphorus (P) (Babikova et al., 2014). A comprehensive understanding of how AMF enhances plant adaptation and mitigates abiotic stress is crucial for sustaining plants, ecosystem management, and climate change mitigation. The prominence of arbuscular mycorrhizal fungi (AMF) parades multifaceted contributions to promoting plant health and productivity, particularly focusing on their influence on plant growth and nutrient absorption in challenging environmental conditions.

Dactylorhiza hatagirea (D. Don), commonly known as Marsh Orchid, is a terrestrial orchid species found in the Himalayan region. This perennial herb is highly valued not only for its medicinal properties but also for its ornamental appeal (Shrivastava and Jain, 2023). The species is characterized by its striking appearance, featuring a rosette of leaves and a dense spike of purple to pink flowers, making it a popular choice for gardens and floral displays. Moreover, its attractive flowers and foliage enhance biodiversity in garden settings, supporting various pollinators and contributing to ecosystem health. Arbuscular mycorrhizal fungi (AMF) form a symbiotic relationship with *D. hatagirea,* playing a crucial role in its growth and development. AMF enhances nutrient uptake, particularly

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phosphorus and nitrogen, which are vital for the plant's robust growth and flowering. This relationship is essential for the plant's survival in nutrient-poor soils, often found in its native habitats. The symbiosis with AMF also aids in the plant's resistance to pathogens and environmental stressors, further enhancing its viability and ornamental value. This mutualistic interaction contributes significantly to maintaining the biodiversity of the regions where *D. hatagirea* is cultivated, as healthy orchid populations support a diverse range of organisms, from soil microbes to pollinators. In this backdrop, this study was taken to quantitatively assess the arbuscular mycorrhiza (AMF) colonization rate in *D. hatagirea* across three locations in the Kashmir Himalaya viz, Gulmarg, Sonamarg and Pahalgam. Additionally, the study examined soil parameters, including temperature, precipitation, pH, organic carbon, phosphorus, potassium, and nitrogen at these sites.

Materials and methods

Collection of root samples

Root samples were obtained from the rhizosphere zone, which is the region surrounding the roots of *D. hatagirea*. Root samples were collected from different sites in Kashmir Himalaya, specifically Pahalgam, Gulmarg, and Sonamarg (Fig. 1, Table 1). The *D. hatagirea* plants were carefully uprooted to collect their root tips, and the sampling depth was 0-20 cm. These Root samples were then stored in sterilized polythene bags and taken to the Plant Pathology and Mycology Laboratory, Department of Botany, University of Kashmir. To maintain the samples' freshness and prevent any potential contamination, the soil-filled polythene bags were stored in a refrigerator at a temperature of 4±1 °C until they were processed for further observations and analysis.

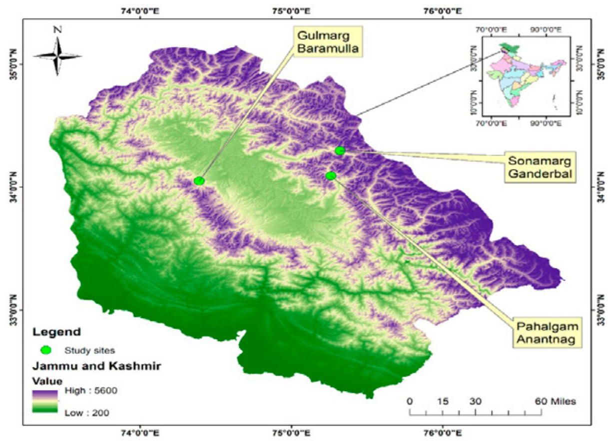

Fig. 1. Map showing the study sites of *Dactylorhiza hatagirea* in Kashmir Himalaya.

Table 1. Salient features of the selected sites in Kashmir Himalaya.

Study sites	Elevation (m a.s.l.)	Latitude (N)	Longitude (E)	Habitat
Pahalgam (Anantnag)	2300	34°05'31"	75°15'36"	Open slopes and forest pastures
Gulmarg (Baramulla)	2500	34°02'58"	74°23'21"	Open slopes and pastures
Sonamarg, (Ganderbal)	2700	34°17'47"	75°19'17"	Open slopes with scree

Assessment of arbuscular mycorrhiza fungi (AMF) root colonization

Root colonization of *D. hatagirea* was conducted following the method outlined by Philips and Hayman (1970) In this study, a meticulous protocol for the assessment of arbuscular mycorrhizal fungi (AMF) colonization in *D. hatagirea* roots was implemented. Root preparation involved the careful washing of roots under running water to eliminate soil, followed by cutting them into 1 cm pieces. Subsequently, the roots underwent treatment with 10% KOH, either through steaming or immersion in a water bath at 100 °C for 30-60 minutes, with subsequent decanting and triple rinsing using distilled water. If root discoloration occurred, an alkaline hydrogen peroxide bleaching treatment was applied, followed by a thorough tap water rinse. Acidification of roots in 2% HCl for 2 minutes was performed until they turned white.

Staining involved immersing roots in a 0.05% trypan blue solution at 90 °C for 8-10 minutes, followed by overnight destaining at room temperature or a 5-10 minutes treatment at 90 °C in 50% glycerol or lactic acid if necessary. Slide preparation included mounting root samples on slides, applying a drop of lactophenol, and covering them with a slip for examination under a dissecting microscope to check for AMF colonization. The assessment phase involved examining plant roots from at least three locations to determine the average colonization percentage. This comprehensive methodology ensures accurate and standardized evaluation of AMF colonization in *D. hatagirea* root samples across different study sites. The percentage of colonization was calculated using the formula:

Soil analysis

Before measuring the pH of the soil samples, the pH meter was calibrated and 20 g of soil from each sample was dissolved in 100 mL of distilled water. The soil suspension was stirred well for half an hour with a magnetic stirrer and then the pH reading of each sample was recorded. The available nitrogen (N), phosphorus (P) and potassium (K) contents of soil samples were determined with the help of Energy Dispersive X-ray spectroscopy (EDS) from the Department of Horticulture, Soil and Leaf Analysis Laboratory, Rajbagh Srinagar and mean temperature, mean precipitation from climate-data.org (Table 2).

Table 2. Soil properties of samples collected from the rhizosphere of *Dactylorhiza hatagirea* assemblages across the study sites in Kashmir Himalaya.

Study sites	Gulmarg	Pahalgam	Sonamarg
Temperature (^{o}C)	21.33	20	17.33
Precipitation (mm)	209.33	201.67	217.33
pH	6.73	6.29	5.52
OC%	7.09	8.27	6.31
Available P (kg h^{-1})	16.39	17.41	25.8
Available K (kg h^{-1})	34.07	35.94	27.15
Available N (mg kg^{-1})	48.51	44.51	35.82

Data analysis

Component Analysis (PCA) was conducted to analyze the variation in environmental and soil variables among the three sites. This analysis aimed to determine whether these sites exhibited distinct differences in terms of these attributes. By reducing the dimensionality of the data, PCA facilitated the visualization of the patterns and relationships between these environmental and soil variables. To explore the overall correlation between different environmental and soil variables, correlation analysis was performed using the Corr plot package in R version 4.2.0. This analysis provided insights into the interrelationships and dependencies among the variables, helping to uncover potential factors driving the observed patterns.

Results

Colonization rate

Our findings reveal significant variations in the percentage colonization of mycorrhizal associations in D. hatagirea roots across the three study sites. Pahalgam (site 1) exhibited the highest percentage of colonization at 78%, (Tab. 2) followed by Gulmarg (site 2) at 73%, and Sonamarg (site 3) displaying the lowest at 57% (Fig. 2; Fig. 3a, 3b).

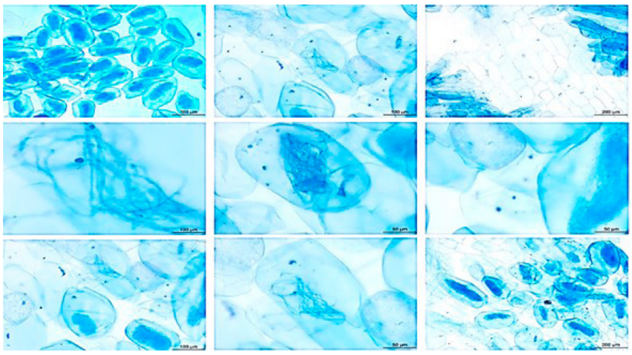

Fig. 2. Root pieces of *Dactylorhiza hatagirea* showing AM fungal infection.

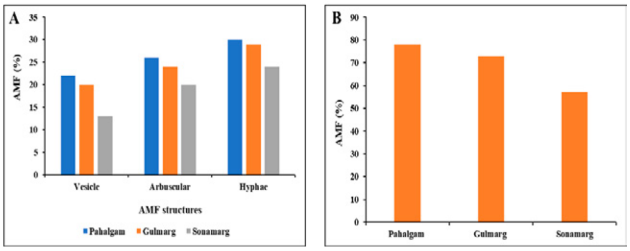

Fig. 3. Percent colonization rate of different AMF structures associated with *Dactylorhiza hatagirea* across different sites (A) and Percent colonization rate of total AMF associated with *Dactylorhiza hatagirea* across different sites (B).

Soil properties of the rhizosphere of *Dactylorhiza hatagirea*

In the Principal Component Analysis (PCA) conducted in our study, the first two PCA axes (PCA 1 and PCA 2) accounted for a total of 69.5% of the overall variation in the environmental and soil variables, with contributions of 49.2% and 20.3%, respectively (Fig. 4). Based on corelation analysis, pH, available nitrogen, available potassium, and organic carbon (OC) exhibited overall positive correlations with each other (Fig. 5). Furthermore, the available phosphorus displayed a negative correlation with available potassium and available nitrogen. Considering these results, the PCA analysis demonstrated that Gulmarg and Pahalgam are relatively like each other concerning these environmental and soil characteristics, whereas Sonamarg exhibited distinct differences in these characteristics when compared to both Gulmarg and Pahalgam. Furthermore, phosphorus exhibits a positive correlation with Sonamarg and a negative correlation with both Pahalgam and Gulmarg. However, other soil parameters show an opposite correlation.

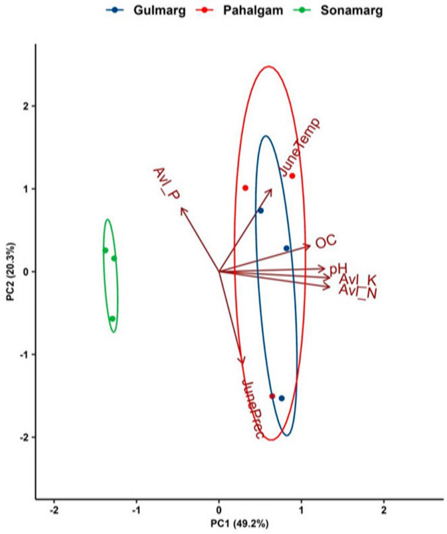

Fig. 4. PCA plot depicting the differences in soil parameters, precipitation and temperature across sampling sites.

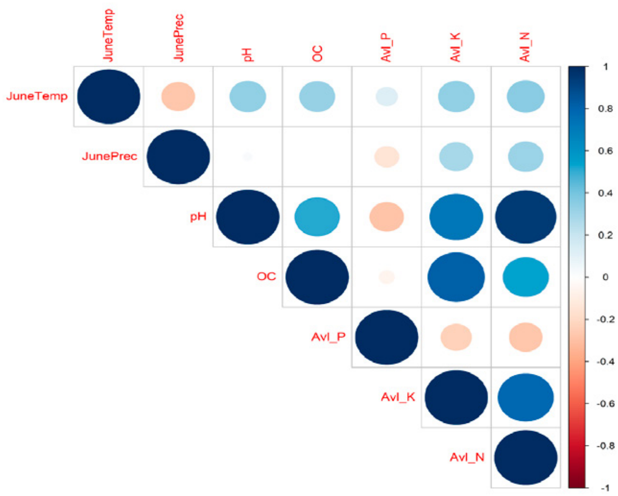

Fig. 5. Corelation plot between soil properties (pH, OC, P, K, and N), precipitation and temperature. The coloured boxes represent significant correlations ($p < 0.01$).

Discussion

Arbuscular mycorrhizal fungi (AMF) engender a suite of physiological enhancements in plant-microbe interactions, particularly in nutrient dynamics (Bhantana et al., 2021; Wahab et al., 2023). The symbiosis involves intricate mechanisms facilitating increased absorption of crucial nutrients, including phosphorus, nitrogen, and zinc, thereby augmenting the availability of essential micronutrients (Upadhayay et al., 2019; Saboor et al., 2021). AMF, through their colonization of the soil matrix, orchestrate transformative effects on both soil physicochemical properties and microbial consortia (Zavahir et al., 2021). The impact of AMF extends beyond mere nutrient acquisition, encompassing a modulation of soil structure characterized by improved aggregation and stability (Lemanceau et al., 2015; Iqbal et al., 2023). Concurrently, the symbiotic presence of AMF elicits a heightened activity of beneficial microorganisms, fostering a multifaceted network that enhances the adaptive capacity of plants to environmental stressors, notably drought and pathogenic challenges. The optimization of AMF colonization emerges as a strategic avenue to fortify plant fitness (Sharma et al., 2021). This scientific endeavour involves a meticulous protocol, as per the method delineated by Philips and Hayman (1970), facilitating precise assessment of mycorrhizal colonization within the root system. This methodological framework encompasses sequential steps, encompassing root preparation, KOH treatment, bleaching, acidification, staining, slide preparation, and systematic assessment of root segments. In the symbiotic nexus between mycorrhizal fungi and plants, a consequential outcome is the facilitation of nutrient and water acquisition by the latter (Chiu and Paszkowski, 2019; Abdalla et al., 2023). This streamlined access translates into accelerated growth rates and heightens physiological vigor, positioning plants on a trajectory of robust health. Additionally, this symbiosis serves as a critical mediator in the mitigation of toxic elements within the soil milieu. The net result of optimizing the symbiotic relationship between AMF and plants holds promise for agronomic and environmental advancements (Berruti et al., 2016). Anticipated outcomes include heightened crop yields, characterized by enhanced nutritional profiles, and the amelioration of soil quality by reducing deleterious elements (Roberts and Mattoo, 2018). Ultimately, this orchestrated synergy contributes to the establishment of a more resilient and ecologically stable ecosystem, exemplifying the intricate interplay between below-ground microbial dynamics and plant health.

In the present investigation, we conducted a study to assess the percentage colonization rate of *D. hatagirea* at three distinct study sites in the Kashmir Himalaya region. This study aimed to quantitatively evaluate the extent of mycorrhizal colonization in *D. hatagirea* within the specified geographic locations. Colonization rates provide valuable insights into the ecological dynamics of plant populations and can be indicative of various environmental factors influencing the distribution and abundance of the species.

Our findings divulge significant variations in the percentage colonization of mycorrhizal associations in *D. hatagirea* roots across the three study sites. Pahalgam (site 1) exhibited the highest percentage of colonization at 78%, followed by Gulmarg (site 2) at 73%, and Sonamarg (site 3) displaying the lowest at 57%. These results underscore the ubiquity of substantial endomycorrhizal associations at all investigated sites. The presence of mycorrhizal associations in *D. hatagirea* roots is of paramount importance, providing key insights into ecological processes such as nutrient acquisition and stress tolerance. The PCA results showed phosphorus positively correlates with the Sonamarg site while other soil parameters showed a positive correlation with the other two sites. It has been shown that soil nutrients, especially higher concentrations of available phosphorus, limit the development of AM fungi (Qin et al., 2020). This symbiotic relationship positively correlates with the overall well-being and ecological adaptability of *D. hatagirea*. However, these mycorrhizal associations are notably sensitive and susceptible to disturbances, including overexploitation, habitat modification, and climate change (Peñuelas and Sardans, 2021). Such stressors may account for the observed decline in *D. hatagirea* populations, rendering it a critically endangered species globally. The present study contributes valuable insights into these intricate interactions, serving as a foundation for the formulation of conservation strategies aimed at safeguarding the threatened *D. hatagirea*. Notably, our results showed an inverse relationship between nutrient accumulation and the AMF colonization rate. Specifically, as indicated by the PCA analysis, certain soil parameters such as pH, nitrogen, potassium, and organic carbon, which positively correlated with each other, were inversely related to the AMF colonization rate observed at the study sites. This inverse relationship suggests that higher levels of these soil parameters might be associated with lower AMF colonization rates, highlighting the complex interactions between soil conditions and mycorrhizal associations in *D. hatagirea.* This finding underscores the importance of considering multiple environmental factors when assessing AMF dynamics and their influence on plant development and biodiversity.

Conclusions

Our findings showed significant variations in the percentage of colonization across the sites, with Pahalgam exhibiting the highest colonization at 78%, followed by Gulmarg at 73%, and Sonamarg displaying the lowest at 57%. These results highlight the prevalence of substantial endomycorrhizal associations at all investigated sites, indicating the importance of these associations in nutrient acquisition and stress tolerance for *D. hatagirea.* There is a positive correlation between phosphorus levels and the Sonamarg site, while other soil parameters exhibit positive correlations with the remaining two sites. Research has demonstrated that soil nutrients, particularly elevated concentrations of available phosphorus, impede the development of arbuscular mycorrhizal (AM) fungi. However, it is crucial to recognize the sensitivity of these mycorrhizal associations to various disturbances, such as overexploitation, habitat modification, and climate change. These stressors may contribute to the observed decline in *D. hatagirea* populations, rendering it critically endangered globally. This knowledge forms a foundation for the development of conservation strategies aimed at safeguarding the threatened *D. hatagirea* and preserving the delicate balance of its ecological niche.

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Author contribution

BAD: Conceptualization; Methodology; Material preparation;Data collection; Data Analysis; Writing – Original Draft; Writing – Review & Editing **RQ:** Conceptualization; Methodology; Material preparation; Data collection; Data Analysis; Writing – Review & Editing. **AHW**: Conceptualization; Methodology; Writing – Review & Editing. **MYB:** Conceptualization; Methodology; Writing – Review & Editing. .

Compliance with ethical standards

This article does not contain any experimentation that violate any ethical standards.

Conflict of interest

The authors declare that they have no conflict of interest.

Data Availability Statement

Data will be available on request.

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