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Dactylorhiza hatagirea: A Critical Issue for Research and Development in Nepal

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Abstract

Dactylorhiza hatagirea (D. don), commonly known as 'Panchaule' in Nepal, is a terrestrial orchid found in temperate to the alpine region. It is valued for its ornamental and medicinal use. It is collected haphazardly from nature due to high economic demand. However, its propagation is limited in nature due to its non-endospermous seeds requiring mycorhizal fungal association for germination. This limitation is leading to the extinction of this orchid from nature and has been enlisted as an endangered and threatened species. Its collection and trade are restricted but have been prioritized for research, conservation, and agro-technology development. As very few research has been reported in *D. hatagirea*, found in Nepal, intensive research, propagation, reintroduction, and commercial cultivation will help control the rhizome collection from nature and meet the economic demand. It will help in the identification and conservation of our local germplasm through its diversity study at the molecular and revenue generation through commercial cultivation under artificial conditions. In this review paper, we discuss the limited research and developments conducted in *Dactylorhiza* at various levels and ways forward for its research, conservation, and utilization. As the plant is valued for its biochemical constituents, modern biotechnological tools such as transcriptomics and metabolomics can be best utilized to explore the opportunities and increase its production and reintroduction through mass propagation for better commercialization and conservation.

Keywords

Dactylorhiza hatagirea flower, herbal medicine orchid, salep

Introduction

D. hatagirea is temperate to alpine, monocotyledonous, perennial, and terrestrial orchid valued for its ornamental and medicinal use. It is a habitat-specific and inherently slow-growing species in nature (Agarwal *et al.,* 2008) and poorly regenerating species because its seeds are microscopic and non-endospermous with undifferentiated embryos. It needs a mycorhizal association for germination, thus posing a significant concern for long-term survival in a natural condition (Warghat *et al*., 2013). The abundance and distribution ranges of orchid species have undergone dramatic declines in recent decades due to human activities, habitat fragmentation, habitat loss due to forest destruction and degradation in the Himalayas where *D. hatagirea* is judged critically endangered (Acharya and Rokaya 2010; Pant and Raskoti 2013). It has a high annual demand of approximately 5000 tons (Badola and Pal 2002), leading to over-exploitation of the species from wild habitat for trade by local inhabitants. Adhikari *et al.* (2018) reported that over-extraction for medicinal purpose and habitat degradation as two significant constraints for the decline in the population of *D. hatagirea* in India. It has been listed in Appendix II by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), vulnerable species listed by the Conservation

Assessment and Management Plan (CAMP) and threatened species by the International Union for Conservation of Nature (IUCN) (Samant *et al*., 2001). The Government of Nepal has prioritized research in *D. hatagirea* for developing agro-technology in Nepal (DPR, 2006), and collection, trade, and rhizome processing has been banned according to Forest Act 1993 and Forest Regulation 1995. Despite government prioritization, as per reports, efforts for its conservation, research, and development, its cultivation has not yet begun as planned. Despite its high economic value, the government has not given much importance for its conservation and production technology development. Most of the research works are concentrated only in the documentation, but the need is to be concentrated on the conservation aspects. Conservation of plant species can be done both *insitu* as well as *ex-situ.* Accessing its existing genetic variability and multiplication through tissue culture can give better alternatives for its *ex situ* conservation and utilization as compared to its protection through the implication of laws restricting its collection and trade. There are various advances in biotechnological tools that can be used to study the genetic variability in the plants; many of them are yet to be employed in *D. hatagirea*. In this review, we are trying to cover the scientific works conducted in *D. hatagirea*and find remaining gaps, which indicate an urgent need for extensive genetic diversity study and establishing an in-vitro propagation technique for *D. hatagirea* found in Nepal for its long term conservation and utilization.

Classification and Morphology

Dactylorhiza hatagirea (D. don) belongs to the Orchidaceae family. The orchids have attracted the admiration of scientists, horticulturists, herbalists, and laypeople alike, and deserve the pride of place in any discussion on flowering plants among the ornamentals. Orchidaceae is believed to be the second-largest family of flowering plants after Asteraceae, with between 21,950 and 26,049 currently accepted species, grouped into 880 genera (World Checklist of Selected Plant Families, 2013). Nepal harbours 451 species of orchid belonging to 104 genera (Rajbhandari, 2015). Central Nepal harbours the highest number of orchid species (69 species) followed by east Nepal (58 species) and west Nepal (33 species) (Acharya and Rokaya, 2010). Among them, *D. hatagirea* is a terrestrial-ground-dwelling perennial herb with erect, hollow and obtuse stem, bears palmately lobed rhizome, and lanceolate leaves with sheathing leaf-base (Fig.1a). The cylindrical and terminal spike bears rosy-purple flowers with green bracts. The inflorescence consists of a compact raceme with 25 to 50 flowers developed from axillary buds. Flowers are 1.7-1.9 cm long with a curved spur, and the dark purple-spotted lip of the flower is rounded and lobed (1 to 5). The structure of orchid flowers is unique among floral plants. The orchid flower typically has an outer whorl of three sepals, an inner whorl of three petals, two identical and one modified (Lip), and a single massive column (the gynostemium, composed of the male stamens attached to the female pistil) in the centre (Fig.1b). The plants store a large amount of water in their palmately lobed tuberous roots (Fig.1c) to survive in arid conditions (Chaurasia *et al.,* 2007).

Distribution

The distribution of *Dactylorhiza* covers most of Europe, temperate Asia, North Africa, Japan, Aleutian Islands, and northern parts of North America with the highest species richness in north-western Europe, though only nine species of *Dactylorhiza* are endemic. *D. hatagirea* is distributed in Nepal, India, Bhutan, China, Mongolia, Pakistan, and Russia (Raskoti and Ale 2009; Sirohi *et al*., 2019). This Himalayan endemic medicinal orchid is also documented in Hindu Kush Himalaya range in terrestrial habitat from Western Himalaya (Afghanistan, Pakistan), India (Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Sikkim), Bhutan, South-East China and Nepal (Hulma, Dolpa, Doti, Kaski, Gorkha, Rasuwa, Sindhupalchok, Dolakha) (Flora of China Editorial Committee, 2009) (Fig. 2). Its habitat is in grassland slopes in the sub-alpine and the alpine zones between 2800 m and 4200 m altitudes above the mean sea level (IUCN, 2004). The altitudinal range of habitat distribution of *D. hatagirea* in Annapurna Conservation Area is reported as 3200 to 3600 meters above sea level with abundance in the slope from 30° to 60° at north-east aspect (Ranpal 2009) and maximum species richness of total medicinal orchid is observed at an elevation

of 1700 m above sea level (Acharya and Rokaya 2010). Hamayun Shaheen *et al.,* (2019) recorded its abundance at 4150 m above sea level in Deosai National Park in Pakistan, and North-West facing steep slopes of Kardang in Ladakh and Shreenagar were found to have *D. hatagirea* as per Jagdish *et al.,* 2018.

In Nepal, studies on orchids are mainly focused on their documentation (Bajracharya *et al*., 2003; Rajbhandari and Dahal 2004; Shakya and Shrestha

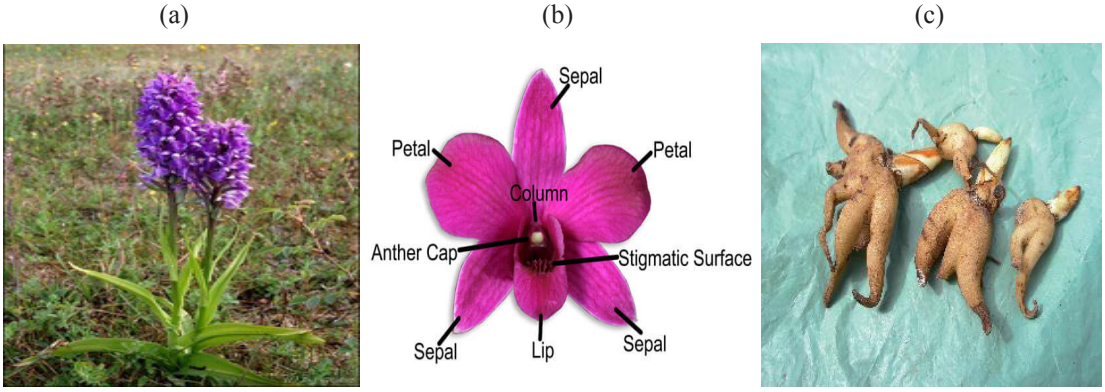

Fig.1. Morphology of *D. hatagirea*(a) aerial part of flowering plants, (b)name of each part of a flower, (c) rhizomes.

2007; Shrestha*et al.,* 2007) and its medicinal uses (Shrestha 2000; Vaidya *et al*., 2000). However, there is less effort on a quantitative study on their distribution pattern along the elevation gradients because information on altitudinal species richness patterns can be highly instrumental for proper management and conservation of species (Grytnes 2003). Chettri and Gupta (2007) documented the presence of *D. hatagirea* at very low (less than 0.2 plant m^2) population density growing above treeline in moist habitat only in Samar Lek site in upper Mustang. Gaire (2014) reported *D. hatagirea* as one of the most potential Medicinal and Aromatic Plants (MAPs) in the Sagarmatha National Park. Bhattarai *et al.,* (2014) studied the availability and conservation status of *D. hatagirea* in Samagaun Village of Gorkha District located within the Manaslu Conservation Area (MCA). They reported a density of 2.18 individuals per·m^2 with frequency and abundance of 81.81% and 2.67 individuals per·m^2, respectively, indicating that the species is threatened in the study area. Pandey *et al.* (2016) also reported overgrazing of domestic animals, over-harvesting, and lack of awareness among the local community as significant causes of *D. hatagirea* decline in then Samagaun Village Development Committee (VDC) of Manaslu Conservation Area (MCA). Khadka *et al.,* (2016) reported a density of 0.276 per m^2 of *D. hatagirea* in Lete VDC of Mustang District, indicating the functional ecological status but unsatisfactory plant diversity, which suggested for immediate necessary actions for the conservation of the diversity in the study area. The high potential of *D. hatagirea* to grow over large areas forming significant continuous distribution boundaries is affected due to anthropogenic activities (Warghat *et al*., 2012). However, Thakur *et al.* (2018) found the enhanced performance of *D. hatagirea* populations driven by disturbances. It suggested that food-deceptive species in small populations tend to reduce the probability of population extinction and have the capability to recover rapidly if conserved on time. It shows the need for systematic planning that incorporates local individuals' participation, prioritization of their views, and cooperation between local institutions, state agencies, researchers, and other stakeholders for the long-term management and commercial utilization.

Phytochemical Study

D. hatagirea is one of the highest valued orchids extensively used in traditional medicine as a farinaceous food and nerve tonic for sick, stomachache, headache, typhoid, effective in chronic diarrhoea, fever, general debility, in treating weakness in children and women, root powder is spread on wounds to control bleeding

Fig. 2. Distribution of *Dactylorhizahatagirea*
Ref.: Raskoti and Ale (2009) and Flora of China Editorial Committee(2009)

(Watanabe *et al*., 2005; Baral and Kurmi 2006; Pant and Raskoti, 2013; Boladi *et al*., 2018 and Nand *et al.,* 2018, Chamoli and Sharan, 2019, Magar *et al*., 2020). It is also considered an alternative source of salep used very commonly in Europe and is considered an essential aphrodisiac plant in Ayurveda, Siddha, Unani literature. Therefore, it is employed to enhance performance and to increase vigour and vitality (Pant and Rinchen, 2012).

It has different spectrums of antibacterial action as evidenced from the result of the various screening tests, indicates that the rhizome part of *D. hatagirea* is more effective than the aerial part against all tested organisms except *E. coli* (Ranpal 2009). At the same time, antifungal compounds are present, assumed from its ability to restrict symbiotic fungal growth inside the orchid cytoplasm (Shimura *et al.,* 2007). Hydroalcoholic extracts were found effective against yeast induced pyrexia in rats (Srohi and Sagar, 2019).

The tubers contain a glucoside, a bitter substance, starch, mucilage, albumen, a trace of volatile oil, and ash (Dutta and Karn 2007). The primary chemical constituents are Dactylorhin A, B, C, D, and E; Dactylose A and B; and two kinds of lipid mixtures along with twelve other compounds in Panchaule found in Nepal (Kizu*et al.,* 1999) (Table 1). Biochemicals from medicinal plants (e.g., Dendrobium plants, Bupleurum, Cactus fruits, *Angelica Sinensis* (Oliv.) Diels, *Aloe barbadensis* Miller, and *Dimocarpus longan* Lour have been found to show prominent bioactivities, including anti-tumour, antioxidant, anticoagulant, antidiabetic, radio-protection effect, antiviral, hypolipidemic and immuno modulatory activities (Xie *et al.,* 2016).

The consumption of salep, prevalent in the traditional medicine of different societies and beliefs, has been effective in improving male

sexual strength (Farnoosh and Riazi, 2007; Esteves *et al*., 2011). Thakur *et al*. (2007) reported improved libido in male rats when injected with aqueous salep extract, which is made from the rhizome of *D. hatagirea*. Yang *et al*., (2006) stated that consumption of natural antioxidants protects sperm cells against oxidative stress-induced by lysed cells and ultimately improves fertility. The therapeutic potential of *D. hatagirea* extract due to high antioxidant properties has also been reported by Sirohi *et al.* (2019). Similarly, the methanolic extract of *D. hatagirea* has been tested in 3T3-L1 cell lines under *in vitro* condition, which shows a non-toxic reaction on the cells indicating the presence of antidiabetic compounds (Alsawalh *et al.,* 2019). The root extract of this orchid has a significant role in reducing blood glucose, lipid, and total protein in experimental diabetic rats, supporting its anti-diabetic properties (Choukarya *et al.,* 2019).

Genetic factors, evolution, geographic variation, environmental conditions (i.e., harvest date, planting time), physiological variations (i.e., organ and leaf position), and developmental stage are known to affect the biosynthesis of the essential oil and other phytochemicals in *Dactylorhiza* (Rodrigues *et al*., 2013 a, b). Thus, there is ample opportunity to produce desired phytochemicals through extensive research to utilize orchids for their varied medicinal uses.

Genetic Diversity

Effective conservation, management, recovery, and utilization of rare and endangered species can be achieved through variability analysis. Genetic diversity assessment provides the basis for *in situ* conservation and sustainable utilization of the plant genetic resources as the marked and robust differentiation among natural populations (Allnutt *et al.,* 2003). Variation in the morphological characters is possible due to variations in topography, elevation, soil fertility, rainfall, and other climatic conditions, which are very specific to Himalaya (Kuniyal *et al.,* 2002). Genetic diversity inherent in plants plays a significant role in the ability of a population to respond adaptively to environmental changes (Ayala and Kiger

Table 1. Chemicals found in *Dactylorhiza hatagirea*

S. N.	Common Name	Chemical Name	Structural Formula	Molecular wt. (g/mol)
1	Dactylorhin A	(2R)-2-β-D-glucopyranosyloxy-2(2-methylpropyl)butanedioic acid bis(4-β-D-glucopuranosyloxybenzyl)ester	$C_{40}H_{56}O_{22}$	888.866
2	Dactylorhin B	(2R,3S)-2-β-D-glucopyranosyloxy-3-hydrosy-2-(2-methylpropyl)butanedioic acid bis(4-β-D-glucopyranosyloxybenzyl)ester	$C_{40}H_{56}O_{23}$	904.865
3	Dactylorhin C	(2R)-2-β-D-glucopyranosyloxy-2-(2-methylpropyl) butanedioic acid	$C_{14}H_{24}O_{10}$	352.336
4	Dactylorhin D	(2R,3S)-2-β-D-glucopyranosyloxy-3-hydroxy-2-(2-mehtylpropyl)butanedioic acid 1-(4-β-D-glucopyranosyloxybenzyl) ester	$C_{27}H_{40}O_{17}$	636.6
5	Dactylorhin E	(2R)-2-β-D-glucopyranosyloxy-2-(2-methyl propyl)butanedioic acid 1-(4-β-D-glucopyranosyloxybenzyl) ester	$C_{27}H_{40}O_{16}$	620.60
6	Dactylose A	1-deosy-1-(4-hydroxyphenyl)-L-sorbose	$C_{12}H_{16}O_6$	256.254
7	Dactylose B	1-deoxy-1-(4-hydroxyphenyl-L-tagatose	$C_{12}H_{16}O_6$	256.254

Source: Kizu *et al.,* 1999

1984). Morphological variability in plants, along with biochemical differences in protein and sugar levels, are essential parameters to study natural populations of rare plants, reflecting an adaptive feature of the species (Bhadula *et al.,* 1981). Molecular markers are the essential tools to study the genetic diversity among different populations, which shows actual genetic differences existing between different populations and can overcome the environmental effects. Therefore, a comparative study on morphological, biochemical, and genetic diversity using molecular marker technique of the populations at different locations of Nepal can give a pleasant scenario of existing genetic variation of this orchid contributing to its conservation and utilization.

Warghat *et al.,* (2012) studied morphological variation in 13 natural population of *D. hatagirea* in Ladakh and reported that phenotypic variation in morphological and horticultural traits could be utilized in its genetic improvement.Germplasm of *D. hatageria* collected from different nine geographical locations of the Garhwal Himalaya was grouped into two clusters based on their similarities and variations in morphology, biochemical parameters, and isoenzyme pattern (Chauhan *et al*., 2014). Localized distribution of *D. hatagirea* (Semwal *et al.,* 2007), habitat fragmentation and population deterioration have increased mating opportunities between closely related individuals, resulting in loss of genetic diversity (Warghat *et al.,* 2013). The low genetic diversity reduced the ability for evolution unless there were opportunities for immigration of new allelic diversity into future populations (Rinchen *et al.,* 2012). Isoenzyme studies supported variability among different populations and suggested esterase isoenzyme as an excellent marker to study intra-population variations in *D. hatagirea* (Bhadula *et al*., 1996). Warghat *et al.* (2012) used 20 Random amplified polymorphic DNA (RAPD) markers and revealed low genetic variation within the population and moderate genetic differentiation among the *D. hatagirea* population in the cold desert of Ladakh. Thakur *et al.,* (2013) also used 33 highly (99%) polymorphic RAPD to study the genetic diversity in *the D. hatagirea* population collected from Himachal Pradesh, India reported the existence of tremendous variability among populations. Warghat *et al.* (2013) revealed a moderate level of genetic differentiation among *D. hatagirea* populations in Nubravalley, Ladakh, and supported the grouping of all 96 sample sizes of nine locations into two collections group using AMOVA of ISSR analysis.

Orchids make up the largest monocot family, but the molecular variation shaping this important biodiversity is still little understood (Givnish *et al.,* 2015). The molecular basis of biodiversity can reside in amino-acid-sequence variation (Nielsen 2005) and regulatory divergence that triggers protein abundance shifts (Wray 2007), and these molecular components may evolve at different rates. The relatively rapid mutation rate and high frequency in the genome have made SSRs be popular markers for population genetics (Hsu *et* al., 2013; Huang *et al.,* 2014 and Tsai *et al*., 2014), hybrid detection (Liao *et al*., 2012), linkage mapping, genetic fingerprinting (Chiou*et al.,* 2012 and Tsai *et al*., 2013), evolutionary history (Ge *et al.,* 2012 and Ge *et al*., 2015), and taxonomy (Ho *et al*., 2014) among various plant species. However, its use in *D. hatagirea* has not yet been documented. De novo transcriptome analysis of *D. hatagirea* revealed the presence of differentially expressed genes governing various metabolic pathways and stress tolerance (Dhiman *et al.,* 2019).There exists a vast opportunity in transcriptomic of *D. hatagirea*, which can be used to reveal various aspects of its uses and values in human life.

Plant Propagation

D. hatagirea is temperate to alpine, monocotyledonous, perennial, and terrestrial orchid valued for its ornamental and medicinal use. It is a habitat-specific and inherently slow-growing species in nature (Agarwal *et al.,* 2008) and poorly regenerating species because seeds are microscopic and non-endospermous with undifferentiated embryos posing a prime concern for long-term survival in a natural condition (Warghat *et al*., 2013). It has a prolonged rate of vegetative propagation and very poor seed germination in nature that is 0.2 to 0.3% (Vij 2002). It has a high annual demand of approximately 5000 tons (Badola and Pal 2002), leading to over-exploitation of the species from wild habitat due

to illegal collection and trade by local inhabitants. In the wake of ongoing habitat loss coupled with global climate change, plant conservation through reserves is not expected to keep pace with the extinction rates projected this century (Swarts and Dixon 2009). An integrated conservation approach is needed to augment in situ conservation, including the recovery, use, and long-term storage of mycorrhizal fungi for symbiotic seed germination (Swarts 2007). Development of a symbiotic germination method of orchid seeds by Knudson (1884-1958), formulating Knudson B and C medium (Knudson 1922, 1946) was the first procedure for *in vitro* propagation of any plant in pure culture (Yam *et al.,* 2009). Therefore, tissue culture can be one of the essential measures in *ex-situ* conservation of terrestrial orchids (Jakobsone *et al*., 2007) through in-vitro mass multiplication as the desirable traits are perfectly preserved in the seedling population (Kesari *et al.,* 2010). Besides, it also helps to produce a significant number of identical clones that can be raised from a single proto-corm, tuber segments, or shoot tip explants (Deb and Pongener 2012) to meet the increasing commercial demand.

The *in vitro* propagation of *D. hatagirea* is very difficult and slow as the process of seed germination to plantlets formation is very lengthy(>1 yr), and very few numbers of *in vitro* raised plantlets can be obtained using green pod culture in M.S. medium (Vij*et al.,*1995). Regeneration using leaf segment, tuber segment culture, shoot bud culture, and green pod culture could not give any positive response (Agarwal *et al.,* 2008). The *in vitro* seed germination is slow not only in *D. hatagirea* but also in the other species of *Dactylorhiza.* In *D. ruthei,* and *D. praetermissa* seeds started germinating after four months of culture, and only 20.0 to 25.0% germination was achieved in Norstog medium after eight months of inoculation (Vaasa and Rosenberg 2004). Rasmussen (1995) reported that most *Dactylorhiza* species require four years from germination to tuber/shoot formation, and two species (*D. majalis*, *D. incarnata*) may take up to 16 years to reach maturity. However, Aggarwal and Zettler (2010) found 100% germination in *D. hatagirea* within ten days after sowing, and plants developed seedlings after three months. Giri and Tamta (2012) also reported better germination on M.S. medium supplemented with peptone (1.0 g/L), morpholino ethane sulfonic acid (1.0 g/L), and activated charcoal (0.1%).

In-vitro protocorm development and mass multiplication of this plant have helped in conservation and increasing biomass using solid M.S. media. Warghat *et al*., (2014) made a successful attempt to culture immature seed embryos of *D. hatagirea* for developing protocorms, shoot regeneration and mass multiplication. They reported seed germination within one week on L.D. and TP039 media supplemented with MnSO4 as an essential component and observed 22%–23% of protocorm formation within 17 days of culture on L.D. medium. The multiple shoot formation was observed in shorter duration on M.S. medium supplemented with 3 mg/L IBA and 1 mg/L Kand the growth and multiplication in 28 to 30 days of incubation followed by successful hardening and transplantation to the greenhouse in the potting mixture.

Popli *et al.* (2016) optimized liquid M.S. media for increasing the biomass of endangered *D. hatagirea* and reported that the growth and development of plantlets with the maximum number of shoots, shoot length, number of roots, maximum root length, and maximum biomass occurred on M.S. medium supplemented with indole-3-butyric acid (IBA) and 6-benzyl amino purine (BAP) within 25 to 32 days of incubation. Liquid medium was reported to increase biomass four times as compared to solid media, which could be used as a platform for its conservation and mass propagation. At the same time, Giri and Tamta (2012) reported using different auxins treatments to obtain *D. hatagirea* at a lower elevation, and it can be enhanced by standardizing more concentration regimes of auxins and suitable environmental conditions at a lower elevation.

There are very few reports on reintroducing *in vitro* propagated species of medicinal orchids to natural habitat (Aggarwal and Zettler 2010; Lesar 2012.), or their cultivation is always collected from the wild for trade. Symbiotic germination has practical merit for both conservation and horticulture, but it remained an under utilized tool for orchids in our context. Aggarwal and Zettler

(2010) reported a successful reintroduction of *D. hatagirea* seedlings grown from mycorrhizal fungi inoculated seeds, which resulted in 100% germination within ten days of sowing, and healthy protocorms were obtained after 40 days, seedlings with well-developed roots, tubers and leaves were obtained after three months. The fungi isolated from the mature plants were characterized using molecular techniques. To substitute for the habitat protection and species recovery, it is crucial to identify and reintroduce over-exposed species through its multiplication using tissue culture.

Conclusion

D. hatagirea has been categorized as endangered and prioritized for its research and conservation by the Government of Nepal. However, there are fewer efforts made to exploit the modern biotechnological tools for its research, conservation, and utilization. *In-vitro* mass propagation of this orchid and their reintroduction in the natural condition can be a good initiative for its *in-situ* conservation. At the same time development of successful agro-technology for its cultivation in commercial-scale under *ex-situ* conditions can fulfil both the medicinal and floricultural sectors. The use of molecular marker technology can give a good idea on the genetic diversity of *D. hatagirea* found in Nepal, and conservation efforts can be directed accordingly. Biomolecules present in the plant make it of high value. Therefore, intensive research on the biochemical constitution of the medicinal plants deserves special attention.There is very little documented information regarding genes related to essential metabolites and their expression. The development of trait-specific molecular markers will help in the identification of diversity in populations. Latest biotechnological tools like transcriptomics and metabolomics can be utilized to understand the biosynthetic pathwaysfor essential metabolites from this orchid. This will help in better utilization for medicinal and therapeutic purposes. If research is focused on this sector, panchaule can be one of the promising high-value products shortly for people living in the mountains of Nepal.

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