Combined effect of PGRs and soil facilitate early flowering of an endangered alpine orchid *Dactylorhiza hatagirea* at lower elevation

Formation of aerial parts, inflorescence or flowers are important adaptive features of alpine plants, making them successful in alpine environment. This is considered as an ubiquitous arctic–alpine adaptation that has evolved independently in all arctic and alpine areas of the world, including the Himalayas. The alpine flora is mainly composed of herbaceous perennials and prostrate shrubs which are well adapted, successful, autochthonous elements of high altitudes[1]. Flower induction in plants is controlled by primary and secondary environmental factors such as photoperiod, temperature, irradiance and water availability. These environmental factors can promote the synthesis of a floral stimulus that is transported through vascular tissues to the shoot apical meristem and subsequently induce flower initiation[2]. Formation of aerial parts, inflorescence or flowers of plants are habitat specific, depends on various microclimatic conditions including altitude and it was difficult to obtain these plant developmental stages at lower elevation due to change in climatic conditions and habitat. However, it is now possible to obtain early sprouting and floral formation of an endangered high altitude medicinal orchid *Dactylorhiza hatagirea* (D. Don) Soo at lower elevation using plant growth regulators (PGRs). Besides, it would also reduce the time for collection of explants required for *in vitro* culture establishment. Early sprouting and flowering at lower elevation provides explants much earlier than at higher elevation.

D. hatagirea (D. Don) Soo, a high altitude terrestrial orchid occurs in temperate to alpine regions (2500–5000 m) in India, Pakistan and Nepal[3], and commonly known as 'Hatajari' in Uttarakhand and 'Salampanja' in Kashmir. It has been categorized as critically endangered[4], rare[5] and listed under appendix II of CITES[6]. The tubers of this species are known to yield a high quality 'Salep' which is extensively used in local medicine as nerve tonic, for its astringent and aphrodisiac properties[7], and is highly nutritive and useful in treating diarrhoea, dysentery and fever and also used as a sizing material in the silk industry[8]. The decoction of the plant is administered during colic pain, tubers are used as expectorant and the extract of the tuber is also used to relieve hoarseness[9]. As the annual consumption of the 'Salep' obtained from this species in India is about 7.38 tonnes (valued at about Rs 50 lakhs), most of it is imported from other countries[7].

Generally, it is difficult to obtain sprouting and different floral formation stages of this orchid at lower altitude because a number of factors are responsible like microclimate of a particular region. Soil also plays a significant role in plant development because it contains a suitable mycorrhizal fungus, which is necessary for the growth of the plant. Terrestrial orchids are associated with mycorrhizal fungi that are considered necessary for seed germination and growth of orchid plants[10–13]. The present study aims at developing a simple and efficient method for inducing early sprouting and flowering of this medicinally important endangered orchid at lower elevation (1990 m).

The alpine soil and tubers of *D. hatagirea* were collected from different alpine meadows of Tungnath (30°30′N–79°15′E; 3300–4200 m)[14], Garhwal Himalaya during October 2007 and brought to the laboratory in perforated polythene bags along with peripheral soil. These tubers were immediately sown in earthen pots at the department nursery containing peripheral soil of the plant. After 18 weeks (last week of January 2008), all the tubers were carefully dug-out from the earthen pots, washed under running tap water, rinsed with distilled water and then air-dried at room temperature (Figure 1 *a*). These tubers were treated with different PGRs (Table 1) for 24 h at 25°C followed by air drying (24 h) (×3). Gibberellic acid (GA_3) and benzyladenine (BA) were obtained from Himedia Laboratory Pvt Ltd, Mumbai, India. Treated tubers were planted in polythene bags and three tubers were used per treatment. The different soils used were non-forest soil collected from nearby site at the department of nursery, district Nainital (1991 m) and alpine soil collected from plant's natural habitat (3300–4200 m). These bags were kept inside the polyhouse and watered regularly. The mean temperature inside the polyhouse was 25 ± 5°C during the experiments. Experiments were observed regularly and data were recorded after three weeks of planting till flower formation. The physio-chemical properties of the soil samples (non-forest and alpine soil) were analysed. Soil texture was determined using sieves of different sizes. Soil pH was measured by glass electrode using 1 : 5

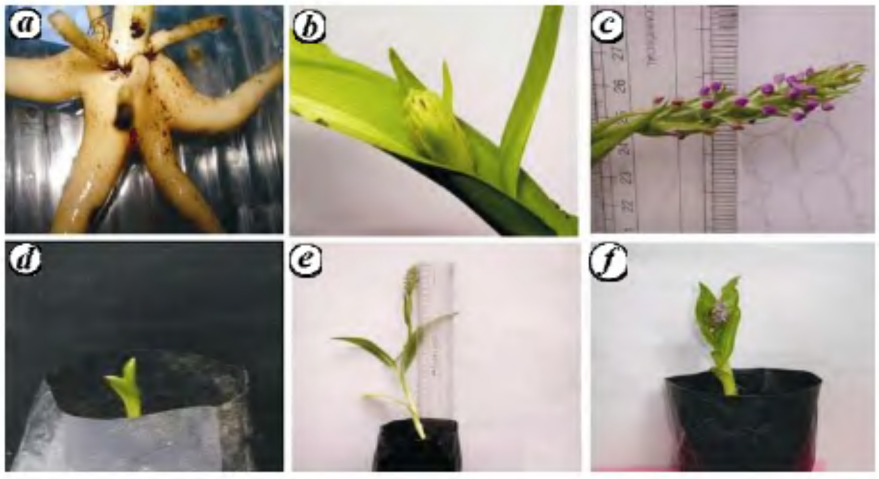

Figure 1. Pretreatments of tubers showing different developmental stages in alpine soil. ***a***, Tuber collected from alpine region in the month of October. ***b*** and ***c***, Initiation and flowering stage, showing in the GA_3 (5.0 μM) treated tuber after 31 days (***b***) and 53 days (***c***) respectively. ***d***, No flowering in control treatment after 44 days. ***e***, Plant flowering as a result of GA_3 (5.0 μM) treatment after eight weeks. ***f***, Plant flowering as a result of BA (2.5 μM) treatment after eight weeks.

Table 1. Effect of PGRs and soil on tubers of *Dactylorhiza hatagirea* in relation to sprouting and flowering at lower altitude (1990 m)

	Non-forest soil					Alpine soil				
PGRs (μM)	Percentage of sprouting	Average time taken for sprouting (days)	Average shoot length (cm)	Percentage of flowering	Average time taken for flowering (days)	Percentage of sprouting	Average time taken for sprouting (days)	Average shoot length (cm)	Percentage of flowering	Average time taken for flowering (days)
Control	33.33 ± 19.24	50.33 ± 26.64	2.75 ± 1.98	00.00 ± 0.00	00.00 ± 0.00	33.33 ± 19.24	44.22 ± 23.34	4.16 ± 2.12	00.00 ± 0.00	00.00 ± 0.00
GA_3 (2.5)	44.44 ± 11.11	30.66 ± 7.19	3.56 ± 0.62	00.00 ± 0.00	00.00 ± 0.00	66.66 ± 19.25	28.88 ± 10.97	6.27 ± 1.33	00.00 ± 0.00	00.00 ± 0.00
(5.0)	55.55 ± 22.22	27.10 ± 12.62	9.19 ± 2.36	00.00 ± 0.00	00.00 ± 0.00	55.55 ± 11.11	20.66 ± 5.55	12.77 ± 2.50	44.44 ± 11.11	53.00 ± 14.18
(10.0)	55.55 ± 11.11	22.44 ± 2.07	4.59 ± 0.78	00.00 ± 0.00	00.00 ± 0.00	77.77 ± 22.22	25.77 ± 6.89	8.14 ± 2.15	00.00 ± 0.00	00.00 ± 0.00
BA (2.5)	66.66 ± 19.29	34.22 ± 2.61	5.38 ± 2.65	00.00 ± 0.00	00.00 ± 0.00	77.77 ± 11.11	25.77 ± 2.62	9.05 ± 2.06	22.22 ± 11.11	34.11 ± 17.69
(5.0)	44.44 ± 22.22	20.11 ± 10.87	3.22 ± 1.63	00.00 ± 0.00	00.00 ± 0.00	66.66 ± 19.24	36.66 ± 11.12	8.33 ± 0.42	00.00 ± 0.00	00.00 ± 0.00
(10.0)	55.55 ± 11.11	24.55 ± 7.05	5.27 ± 1.47	00.00 ± 0.00	00.00 ± 0.00	55.55 ± 22.22	20.55 ± 7.89	5.61 ± 1.45	00.00 ± 0.00	00.00 ± 0.00

± Standard error (SE). Data were recorded after sprouting till eighth week.

proportions of soil and water[15]. Organic carbon[16] was determined and the factor 1.724 was used to convert the organic carbon into soil organic matter. The total nitrogen was analysed by Kjel auto N analyser. Available phosphorus (P) and available potassium (K) were analysed by following the methods given in ref. 17. Data were subjected to statistical analysis and standard error (SE) and correlation was calculated following standard method[18].

Sprouting started 20 days after planting tubers in both types of soil in all the treatments including control and it took up to 50 days for all the tubers to sprout (Table 1). In non-forest soil planted tubers, the maximum sprouting was 66.66% whereas it was up to 77.77% in alpine soil planted tubers. Although BA (2.5 μM) was found to be most effective for sprouting in non-forest soil planted tubers, it was GA_3 (5.0 μM) which enhanced average shoot length up to 9.19 cm (Table 1). In control, sprouting was only 33.33% and tubers took a long time to sprout (50 days), shoot length was also very short (2.75 cm) in control compared to other treatments in non-forest soil (Table 1). In non-forest soil, GA_3 (5.0 μM) showed a significant correlation between average time taken for sprouting and sprouting percentage (at 0.05) and average time taken for sprouting and average shoot length (at 0.01). However, average shoot length showed a significant correlation between sprouting percentage (at the 0.05%) and average time taken for sprouting (at the 0.01%). Later, the seedlings that emerged from non-forest soil tubers did not survive and dried few weeks after sprouting. This may be because orchids are mycoheterotrophic during their seedling stage and in many species, the dependency on fungi as a carbohydrate source prolongs till adulthood[19]. In alpine soil tubers, the maximum sprouting percentage (77.77) was recorded in GA_3 (10.0 μM) and BA (2.5 μM) treated tubers. The average time taken for sprouting showed a significant correlation with sprouting percentage in GA_3 (2.5 μM) (at the 0.01%) and BA (10.0 μM) (at the 0.05%). The average shoot length also showed a significant correlation with sprouting percentage (at the 0.01%) in GA_3 (2.5, 10 μM) (at the 0.05%). Here, further growth of seedlings was good and they achieved flowering stage. Flowering was 44.44% in GA_3 (5.0 μM) followed by 22.22% in BA (2.5 μM) treated tubers within 34 and 53 days respectively (Table 1 and Figure 1 *b*, *c*, *e*, *f*). Other treatments including control (Figure 1 *d*) were not able to induce flowering. The flowering percentage was significantly correlated (at the 0.01%) with sprouting percentage and the days taken for flowering (at the 0.05%) only in BA (2.5 μM) treatment. Achievement of flowering stages at lower elevation may be due to the combined effect of PGRs and soil because when PGRs were used alone, the tubers failed to flower. When the PGR treated tubers were planted in alpine soil instead of non-forest soil, only then tubers were able to flower.

The physiochemical properties of different types of soil, i.e. non-forest and alpine soil are given in Table 2. The two types of soil showed different physiochemical properties. Soil texture was sandy loam in both types of soil but the non-forest soil was alkaline (7.7 pH) whereas alpine soil was acidic (5.7 pH). Differences were also observed in their primary nutrients (NPK). Total organic matter, carbon and C : N ratio was higher in alpine soil compared to non-forest soil (Table 2).

In both the experiments, sprouting started during February–March whereas in its natural habitat, it starts during May–June. Similarly, flowering stages were observed in April–May in the present study whereas in nature the floral formation starts during July–August. Both the stages, i.e. sprouting and flowering, were two months earlier at lower elevation compared to its natural habitat (at higher altitude). This may be due to the collective effect of PGRs, soil as well as temperature and altitude. PGRs influence many diverse developmental pro-

Table 2. Physiochemical properties of different types of soil

Parameters	Non-forest soil	Alpine soil
Soil texture (%)		
Sand	68	67
Silt	27	15
Clay	5	18
Soil pH	7.7	5.7
Primary nutrients		
Nitrogen (%)	0.72	0.46
Available phosphorus (ppm)	0.0067	0.0004
Available potash (ppm)	0.0169	0.028
Total organic matter (%)	7.73	12.5
Carbon (%)	4.483	7.256
C : N ratio	6.227	15.77

Soil analysis was done within a week after collection.

cesses ranging from seed germination to root, shoot and flower formation[20]. In this study, PGRs alone were not sufficient to induce flowering at lower elevation; alpine soil was also required to induce flowering. Also, alpine soil alone could not induce flowering in the control (Table 1). Besides these differences, alpine soil must contain mycorrhizae necessary for its growth and further development because it has been collected from the rhizosphere region of the plant and its surroundings in its natural habitat. On the basis of available literature[19], it was hypothesized that alpine soil must contain a suitable fungus (data not shown) which will be helpful in plant development.

This type of study is the first attempt and could be a successful effort at growing this endangered alpine orchid at lower elevation. This species is habitat specific and inherently slow growing in nature[7] and collection of its germplasm is difficult as it is an endangered medicinal plant[21]. Research work is under progress for getting further stages like pod formation and seed maturation at lower elevation.

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