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SUBSTRATUM ANALYSIS OF SOME THERAPEUTICALLY SIGNIFICANT AND/OR ENDANGERED ORCHIDS OF SHIMLA HILLS (HIMACHAL PRADESH), NORTHWESTERN HIMALAYAS AND THEIR CONSERVATION

Roshan Lal and Promila Pathak

Orchid Laboratory, Department of Botany, Panjab University, Chandigarh- 160 014, U.T., India

Abstract

Shimla hills located at an altitude range 1600-2800 m amsl, are biodiversity rich zones in NorthWestern Himalayas. Terrestrial orchids have special preference for this area because of its vast vegetation ranges, forest cover, and favourable climate. Orchids are generally highly adapted to form specific biological interactions with other organisms; flowers are usually specialized to specific pollinators and all species rely on mycorrhizal symbiosis with specific groups of fungi. They have been used in many parts of the world in traditional healing system as well as in the treatment of a number of diseases since the ancient time. Orchids are considered as indicator species of habitat disturbances. Recently, these plants are listed amongst the most threatened taxa globally due to increasing anthropogenic threats, inherent rarity, and specific conservation needs. The most commonly cited threats affecting orchids are deforestation, logging, forest fire, road construction, and the expansion of forest plantations and agriculture, and their over-collection for the ornamental, medicinal, food, and trade. The present investigation deals with substratum analysis of some therapeutically significant and/or endangered orchids from Shimla and adjacent hills. It was observed that these plants grow in a variety of habitats, having different soil types with slightly acidic pH, and with species specific nutrient requirements. The information obtained after soil analysis will indicate about the nutritional requirements of species, and this information can later be utilized for formulation of species specific nutrient media for their *in vitro* mass propagation and conservation programmes.

Introduction

THE STATE of Himachal Pradesh (30°22'40"N to 33°12'40"N latitudes and 75°45'55"E to 79°04'20"E longitudes) covers the parts of Trans and NorthWestern Himalayas. It is bounded by Tibet in East, Jammu and Kashmir in the North, Uttarakhand in the SouthEast, Haryana in the South, and Punjab in the West. The family Orchidaceae is one of the largest families of flowering plants and orchids are almost globally distributed with 28,484 species (Govaerts *et al*., 2017). In India, about 1256 species of orchids, belonging to 155 genera have been recorded (Singh *et al*., 2019). While the orchid diversity is relatively very less in Himachal Pradesh, it supports natural, unique, and socio-economically important orchids (Chauhan, 1999; Deva and Naithani, 1986; Kumar *et al.,* 2018; Kumar *et al.,* 2019; Prakash and Pathak, 2019; Sharma and Samant, 2017). The state is represented by 85 species of orchids (Vij *et al*., 2013) and a few more are added every year. They are found almost everywhere except in the hot desert and Antartica. Depending upon their habitat, they can be classified into following different categories namely terrestrials (*Calanthe tricarinata*, *Dactylorhiza hatagirea*, *Epipactis helleborine etc.*), epiphytes (*Aerides multiflora*, *Dendrobium amoenum etc.*), and achlorophyllous saprophytes (*Aphyllorchis montana*, *Erythrorchis ochobiensis etc.*), or subterranean (*Rhizanthella gardneri*).

Most orchids are narrowly distributed in specific habitats because of their mycorrhizal specificity, pollinator specialization, and limited seed germination rate. Compared to plants from other families, orchids are extremely susceptible to habitat disturbance. These plants are popular for exhibiting wide range of pollination mechanisms (Darwin, 1862; Micheneau *et al*., 2009, Pal *et al.,* 2019). Many orchid species attract pollinators with forms of deception including food deception, brood-site imitation, shelter imitation, rendezvous attraction, and sexual deception (Buragohain and Chaturvedi, 2016; Jersakova *et al*., 2006). Orchid roots are usually thick and succulent, produce a large biomass, but have a very few root hair (Hew and Yong, 2004). The presence of mycorrhizal fungi can increase the absorbing surface area of those roots (Dearnaley and Cameron, 2017).

Altitude, soil acidity, and certain habitat types were found to be the most important factors in determining orchid distribution (Tsiftsis *et al*., 2008). Terrestrial orchids obtain nutrients mainly from the soil, while sources for epiphytic orchids can also include atmospheric dry/wet depositions, solid substrates (such as bark or litter), and nitrogen fixation by microorganisms (Benzing, 1990; Reich *et al*., 2003). The sites suitable for seedling establishment of *Cypripedium calceolus* were characterized as having relatively extensive moss cover, less vascular plant cover, more moisture, and better light conditions (Kull, 1998). Nutrients are important

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factors that control plant growth and development. For example, nitrogen-deficiency can decrease protein synthesis, growth rates, and productivity (Amancio and Stulen, 2004). *Cypripedium cordigerum* occupied substratum with slightly acidic soil pH and higher contents of organic carbon, nitrogen, phosphorus, and potassium and this substratum was best suited for its growth and development (Verma *et al*., 2014).

The orchids are of great value in ornamental, medical, trade industry, and evolutionary research. Their therapeutic uses include *Dactylorhiza hatagirea* (used as tonic and as aphrodisiac); *Eulophia dabia* (used as stomach tonic, aphrodisiac, and blood purifier); *Spiranthes australis* (anti-cancer or anti-tumour) (De *et al*., 2016; Jalal *et al*., 2008; Pathak *et al*., 2010). The various indigenous uses of presently studied plant species are given in Table 1. Most formal, global orchid trade is in artificially propagated cut-flowers and plants grown under controlled conditions. Between 1996 and 2015, Taiwan and Thailand were the largest exporters, with most plants sent to South Korea (40%), USA (27%), and Japan (20%), respectively (UNEP-WCMC, 2017). Thailand is the largest exporter of orchid cut flowers to

Table 1. Species, locality, habit, habitat, altitude range, associated vegetation, and indigenous uses of presently studied orchids of Shimla hills.

Species	Locality	Habit and habitat	Altitude range (m amsl)	Associated vegetation	Indigenous uses
Calanthe tricarinata Lindl.	Narkanda	Terrestrial; occupied shady, riverine, moisture rich forest floor.	2400-2650	Mosses, ferns, liverworts, orchids (*Goodyera repens*), and mixed forests.	Used to cure sores, eczema, and as aphrodisiac (Pant, 2013).
Crepidium acuminatum (D.Don) Szlach.	Summer hill	Terrestrial; occupied shaded to semi-shaded, litter rich, low moisture forest floor.	2100-2250	Mosses, liverworts, orchids (*Herminium lanceum, Liparis rostrata,* and *Satyrium nepalense*), and mixed forests.	Used for burns, as a tonic, used to treat bronchitis, fever, tuberculosis, and weakness and used as one of the ingredients of *Ashtavarga* of Ayurveda (Pant, 2013); used as febrifuge, and spermopiotic (De *et al*., 2015).
Epipactis helleborine (L.) Crantz	Kufri	Terrestrial; occupied shaded to semi-shaded, meadows, litter and humus rich, moist forest floor.	2450-2700	Mosses, ferns labiates, umbellifers, sedums, orchids (*Cephalanthera longifolia*, *Cypripedium cordigerum,Goodyera repens*), and mixed forests.	Used to treat insanity, gouts, headache, and used as an aphrodisiac and used to cure fever; as blood purifier (Barman *et al*., 2016).
Goodyera repens (L.) R.Br.	Kufri	Terrestrial; occupied shaded to semi-shaded, moisture rich forest floor.	2450-2700	Mosses, ferns, liverworts, orchids (*Cephalanthera longifolia*, *Cypripedium cordigerum*, *Epipactis helleborine*, *Malaxis muscifera*), and mixed forests.	Plant paste externally applied in syphillis, plant extract is taken as a blood purifier (Linthoingambi *et al*., 2013).
Herminium lanceum (Thunb. ex Sw.) Vuijk	Water Catchment WildLife Santuary	Terrestrial; occupied, semi-shaded, degraded, dry exposed surface, along road embankments, and forest borders.	1950-2300	Mosses, potentillas, scrophs, grasses, orchids (*Crepidium acuminatum, Habenaria latilabris*, *Liparis rostrata*, and *Satyrium nepalense*), and mixed forest.	Used to treat cold and fever, rheumatism, typhoid fever, hernia, sores, eczema, snake bites, and for reducing swelling and pain. The plant extract is given to cure suppressed urination (Prakash and Pathak, 2019).
Neottia listeroides Lindl.	Taradevi	Achlorophyllous/ subterranean; occupied shaded, litter or humus rich, moist forest floor.	1800-1950	Mosses, ferns liverworts, labiates, legumes, scrophs, and orchid (*Goodyera biflora*), and mixed forests.	-

Table 2. Various physico-chemical parameters of soil samples of presently studied orchid species.

Species	pH	Electrical conductivity (mS)	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulphur (%)
Calanthe tricarinata Lindl.	6.88	0.44	3.352	0.986	0.140	BDL
Crepidium acuminatum (D.Don) Szlach.	6.72	0.33	7.056	1.435	1.008	BDL
Epipactis helleborine (L.) Crantz	6.35	0.08	1.022	0.924	0.064	BDL
Goodyera repens (L.) R.Br.	6.96	0.19	5.850	1.365	0.750	1.247
Herminium lanceum (Thunb. ex Sw.) Vuijk	6.89	0.60	4.110	1.101	0.167	BDL
Neottia listeroides Lindl.	6.73	0.30	1.135	0.632	0.050	BDL

BDL = Below detection limit

India devoting 80.67% of total import followed by Netherlands (15.54%), New Zealand (2.29%), and China (1.5%) (De, 2020).

The changing environmental conditions, land use patterns, over grazing, over-exploitation, and expanding urbanization have resulted in shrinkage and degradation of natural habitats causing threat to floristic diversity in general and orchids in particular. Kull and Hutchings (2006) presented a comparative analysis of decline in orchid range in two highly contrasting European countries-Estonia, which had a very low human population density and much conserved semi-natural habitat, and U.K., where population density was much higher and remaining semi-natural habitat was rapidly loosing species. Under same time period, there had been much higher orchid decline in U.K. Orchids may be particularly vulnerable to over-harvest because many species have a limited range and/or occur at low densities due to a variety of interacting factors such as recent speciation, specialized pollination mechanisms, habitat specificity, and the restricted distribution of mycorrhizal symbionts (Buragohain and Chaturvedi, 2016; Manoharachary, 2019; McCormick and Jacquemyn, 2014; Swarts and Dixon, 2009). The International Union for Conservation of Nature (IUCN) has a Species Survival Commission (SSC) with a well defined preservation programme for the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Under this provision, orchids are treated as protected species. More than 70% of the orchid species are listed on CITES. India became a party to CITES since 1976 and is also a signatory of the Convention on Biological Diversity (CBD) since 1992. The latest update of the IUCN Global Red List (IUCN, 2020), included 88 new assessments for orchids, and this brings the total number of orchid species that have been assessed to 1641 (*ca*. 6% of orchids). Five of these are extinct and 747 are threatened, 197 Critically Endangered (CR), 355 Endangered (EN), and 195 Vulnerable (VU). Eighty seven genera and 575 species have been assessed as Near Threatened (NT) and Least Concern (LC), respectively. In the first decade of the 21st century, the proportion of threatened orchids on the Red List ranged from *ca*. 80 to >90%, but this is largely a result of selecting predominantly high-profile species due to perceived threat. There are three prominent methods of conservation of genetic resources of orchid

Table 3. Analysis of mineral contents of soil samples of presently studied orchid species.

Species	Sodium ($mgkg^{-1}$)	Potassium ($mgkg^{-1}$)	Calcium ($mgkg^{-1}$)	Magnesium ($mgkg^{-1}$)	Nitrate ($mgkg^{-1}$)	Phosphate ($mgkg^{-1}$)
Calanthe tricarinata Lindl.	8.73	66.88	213.33	105.33	13.86	BDL
Crepidium acuminatum (D.Don) Szlach.	14.33	73.53	133.33	72.83	7.25	BDL
Epipactis helleborine (L.) Crantz	8.01	70.61	160.00	56.66	1.11	0.65
Goodyera repens (L.) R.Br.	10.98	66.51	106.66	97.16	1.80	BDL
Herminium lanceum (Thunb. ex Sw.) Vuijk	13.88	64.95	320.16	81.00	11.51	0.33
Neottia listeroides Lindl.	13.93	64.40	307.00	97.33	2.66	0.81

BDL = Below detection limit

Table 4. Composition of modified M medium for presently studied orchid species.

Species	$MgSO_4.7H_2O$ (gml^{-1})	KNO_3 (gml^{-1})	$(NH_4)SO_4$ (gml^{-1})	NaH_2PO_4 (gml^{-1})	$Ca(NO_3)_2.4H_2O$ (gml^{-1})	Minor (mlL^{-1})	Vitamins (mlL^{-1})	Sucrose (gml^{-1})	EDTA (mlL^{-1})	Agar (gml^{-1})
Calanthe tricarinata Lindl.	0.021	0.013	1.40	0.021	0.043	1.0	1.0	33.52	3	8.5
Crepidium acuminatum (D.Don) Szlach.	0.014	0.015	10.08	0.003	0.027	-do-	-do-	70.56	-do-	-do-
Epipactis helleborine (L.) Crantz	0.011	0.014	0.64	0.002	0.032	-do-	-do-	10.22	-do-	-do-
Goodyera repens (L.) R.Br.	0.019	0.013	7.50	0.002	0.021	-do-	-do-	58.50	-do-	-do-
Herminium lanceum (Thunb. ex Sw.) Vuijk	0.016	0.013	1.67	0.003	0.064	-do-	-do-	41.10	-do-	-do-
Neottia listeroides Lindl.	0.019	0.013	0.50	0.003	0.061	-do-	-do-	11.35	-do-	-do-

$MgSO_4.7H_2O$ (Mg source); KNO_3 (K source); $(NH_4)SO_4$ (N source); NaH_2PO_4 (Na source); $Ca(NO_3)_2.4H_2O$ (Ca source); and sucrose (Carbon source).

Table 5. Composition of modified MS medium for presently studied orchid species.

Species	$MgSO_4.7H_2O$ (gml^{-1})	KNO_3 (gml^{-1})	NH_4NO_3 (gml^{-1})	KH_2PO_4 (mgl^{-1})	$CaCl_2.2H_2O$ (gml^{-1})	Minor (mlL^{-1})	Vitamins (mlL^{-1})	Sucrose (gml^{-1})	EDTA (mlL^{-1})	Agar (gml^{-1})
Calanthe tricarinata Lindl.	0.021	0.013	1.40	-	0.043	1.0	1.0	33.52	10	8.5
Crepidium acuminatum (D.Don) Szlach.	0.014	0.015	10.08	-	0.027	-do-	-do-	70.56	-do-	-do-
Epipactis helleborine (L.) Crantz	0.011	0.014	0.64	0.13	0.032	-do-	-do-	10.22	-do-	-do-
Goodyera repens (L.) R.Br.	0.019	0.013	7.50	-	0.021	-do-	-do-	58.50	-do-	-do-
Herminium lanceum (Thunb. ex Sw.) Vuijk	0.016	0.013	1.67	0.06	0.064	-do-	-do-	41.10	-do-	-do-
Neottia listeroides Lindl.	0.019	0.013	0.50	0.16	0.061	-do-	-do-	11.35	-do-	-do-

$MgSO_4.7H_2O$ (Mg source); KNO_3 (K source); $(NH_4)SO_4$ (N source); KH_2PO_4 (P source); $CaCl_2.2H_2O$ (Ca source); and sucrose (Carbon source).

species; legislative measures; *in situ* conservation in Sanctuaries/Reserves; and *ex situ* conservation in Orchidaria/Botanic gardens by cultivation. Hence, there is an urgent need to conserve these species by tissue culture techniques (Bhatti *et al.,* 2017; Kaur *et al.,* 2017; Mohanty and Salam, 2017; Pathak *et al.,* 2001; Vasundhra *et al.,* 2019). The application of tissue culture techniques, which help in achieving rapid mass propagation of desired genotypes, can open new possibilities in their conservation and commercialization.

The basic problem with terrestrial orchid propagation is low probability of seed germination. Terrestrial orchids unlike their epiphytic counterparts, are difficult to germinate *in vitro*. *In vitro* seed germination of orchids is recorded for some species including *Arundina graminifolia* (Sibin *et al*., 2014); *Bletia purpurea* (Dutra *et al*., 2008); *Cephalanthera longifolia* (Padder *et al*., 2014), *Coelogyne fimbriata* (Anuprabha and Pathak, 2019); *Cymbidium giganteum* (Ghosh *et al*., 2014; Hossain *et al*., 2010); *Cymbidium macrorhizon* (Vij and Pathak, 1988); *Dactylorhiza hatagirea* (Vij *et al.,* 1995); *Dendrobium ovatum* (Gurudeva, 2019); *Eulophia promensis* (Hossain, 2015); *Herminium lanceum* (Singh and Babbar, 2016); *Paphiopedilum spicerianum* (Chen *et al*., 2015); *Phaius tankervilleae* (Vimal *et al*., 2018); *Satyrium nepalense* (Chauhan *et al*., 2010); *Spathoglottis plicata* (Sebastinraj and Muhirkuzhali, 2014); *Smithsonia maculata* (Decruse and Gangaprasad, 2018); *Vanda spathulata* (Lekshmi and Decruse, 2018); *Vanda tessellata* (Madhavi and Shankar, 2019).

As only a few species have so far been studied for their soil profiling and nutritional requirements (Bowles *et al*., 2005; Devi *et al*., 2018; Kull and Hutchings, 2006; Kusum *et al*., 2013; Ors *et al*., 2011; Ramsay and Stewart, 1998; Tsiftsis *et al*., 2008; Verma *et al*., 2014; Vij *et al*., 1998), more attention is needed for terrestrial orchids for understanding their nutritional requirements

for *in vitro* asymbiotic seed germination and accordingly devising the nutrient formulations. Keeping in view the above, presently, substratum analysis of six orchid species was carried out so as to collect information about the type of soil (substrate) and nutrient contents of the soil, and subsequently using this information for modifying standard nutrient media for successful *in vitro* asymbiotic seed culture of these species.

Material and Methods

Substratum (soil profile) is an important aspect for the terrestrial orchids. During the present investigation, substratum analysis with different aspects such as habit, habitat, associated vegetation, physico-chemical parameters including soil pH, electrical conductivity, and nutrient contents of six orchid species namely *Calanthe tricarinata* Lindl., *Crepidium acuminatum* (D.Don) Szlach., *Epipactis helleborine* (L.) Crantz, *Goodyera repens* (L.) R.Br., *Herminium lanceum* (Thunb. ex Sw.) Vuijk, and *Neottia listeroides* Lindl. were studied.

Study Area

The present study was carried out in Shimla hills of Himachal Pradesh, NorthWestern Himalaya, which have very rich floral and faunal diversity. Geographically, Shimla hills are located at an altitude 1600-2800 m amsl with 31°11'16''N latitude and 77°20'30''E longitude. The various orchid species were studied from different localities including Narkanda, Summer hill, Water Catchment Wildlife Sanctuary, Kufri, and Taradevi. Soil samples were collected from the field after clearing the litter or humus layer by digging down (10 cm) at three different nearby zones and then, final samples were prepared.

Preparation of Soil Extract (S.E.)

The collected soil samples were oven dried at 40-45°C, followed by grinding with the help of mortar and pestle and filtered through sieve. CHNS elemental analysis (Carbon, Hydrogen, Nitrogen, and Sulphur) was done with dry and powdery soil samples while rest analysis was performed with the help of soil extract. Soil extract (S.E.) was prepared with 20 gm soil dissolved in 100 ml distilled water, kept on shaker overnight followed by filtration through Whatman's filter paper and further, soil analysis for pH, electrical conductivity, and nutrients (Sodium, Potassium, Calcium, Magnesium, Nitrate, and Phosphate) was done.

Soil Analysis

The various physico-chemical parameters of the soil samples were analysed, using different techniques (Chand *et al*., 2011; Trivedy *et al*., 1987). The soil pH and electrical conductivity (EC) were measured with digital electrical pH meter and digital electrical conductivity meter. CHNS elemental analysis was done for the analysis of Carbon (C), Hydrogen (H), Nitrogen (N), and Sulphur (S) contents. Sodium (Na) and Potassium (K) were analysed by flame-photometer, Calcium (Ca) and Magnesium (Mg) by titrimetrically, and Nitrates (NO^{3-}) and Phosphates (PO_4^{3-}) by spectrophotometer, respectively.

Results and Discussion

The substratum analysis conducted in six orchid species namely *Calanthe tricarinata* Lindl., *Crepidium acuminatum* (D.Don) Szlach., *Epipactis helleborine* (L.) Crantz, *Goodyera repens* (L.) R.Br., *Herminium lanceum* (Thunb. ex Sw.) Vuijk, and *Neottia listeroides* Lindl. showed that orchids have different habits, habitats, altitudinal range, and associated vegetation (Table 1). These plants occupied a region varied from shaded to semi-shaded, moisture rich, open grasslands, and dry rocks. They were observed growing along with mosses, lichens, and liverworts which have high water retention capacity and prevent dryness. Beside this, these plants were distributed on road embankments and borders of mixed forests. According to Sanford (1974), the species that generally inhabit shady to semi shady habitats require moist and humus rich soils with less exposure to direct sunlight. The soil texture varied from loamy to sandy and sandy-loam. Species which require high moisture occupied loamy soil (*C. tricarinata* and *G. repens*) and species with low moisture contents and more aeration occupied sandy (*C. acuminatum* and *H. lanceum*), and sandy-loam soil (*N. listeroides*). Naik *et al*. (2009) demonstrated that the rate of mineral uptake by orchid roots is relatively low as compared to the other crop plants. Such observations clearly indicates that not only one or two soil characteristics influence the germination of orchid seeds in a given soil/locality; all factors (abiotic and biotic) are of high importance in making microclimate congenial for initial orchid seed germination and successful seedling establishment, thereafter. The soil pH was slightly acidic (6.35-6.96) for presently studied species (Table 2). It was highly acidic for *E. helleborine* and nearly neutral for *G. repens*. Orchids required greater uptake of nutrients and water from the substratum, so they thrive in soil with pH ranging between 6.0-6.9 (Ito, 1955; Knudson, 1945; Raguvanshi *et al*., 1986). The seedlings are less sensitive to pH variations and pH is critical only during early stages of orchid seed germination (Knudson, 1951). According to Davidson (1960), mineral contents of soil are more critical, in orchid growth, than its pH. Electrical conductivity which measures the concentrations of free soluble ions present in the soil was high for *C. tricarinata*

and *H. lanceum* indicating thereby that these species need more soluble ions as earlier recorded by Vij *et al*. (1998). However, *E. helleborine* occupied the least conducive soil followed by *G. repens*.

The requirements of macronutrients which are essential during early as well as later stages of development vary from species to species. Orchids can adapt to varied nutritional regimes and are even capable of survive under nutritionally marginal conditions (Stoutamire, 1974). Poole and Sheehan (1982), however, demonstrated that the nutrient requirement varies with the genera in orchids. In the present study, these plants had Carbon, Hydrogen, Nitrogen, Calcium, and Potassium rich substratum and low Sodium, Sulphur, and Phosphate contents. Carbon, Hydrogen, and Nitrogen were high for *C. acuminatum* and *G. repens* while *E. helleborine* and *N. listeroides* had low carbon, hydrogen, and nitrogen contents (Fig. 1). Nitrogen rich substratum has often been considered conducive to orchid growth (Sheehan, 1961). Presently, Sulphur was found to be below detection limit for all the species except *G. repens*.

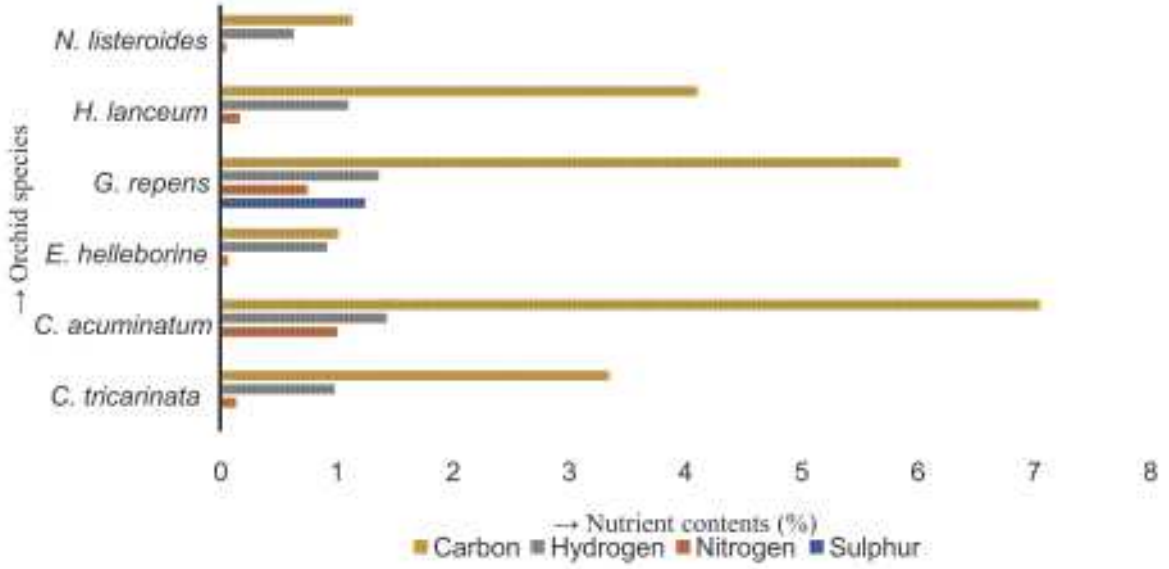

Fig. 1. CHNS elemental analysis of soil samples of presently studied orchid species.

H. lanceum and *N. listeroides* inhabited sodium rich substrata while *C. tricarinata*, *C. acuminatum*, and *E. helleborine* occupied Potassium rich substrata

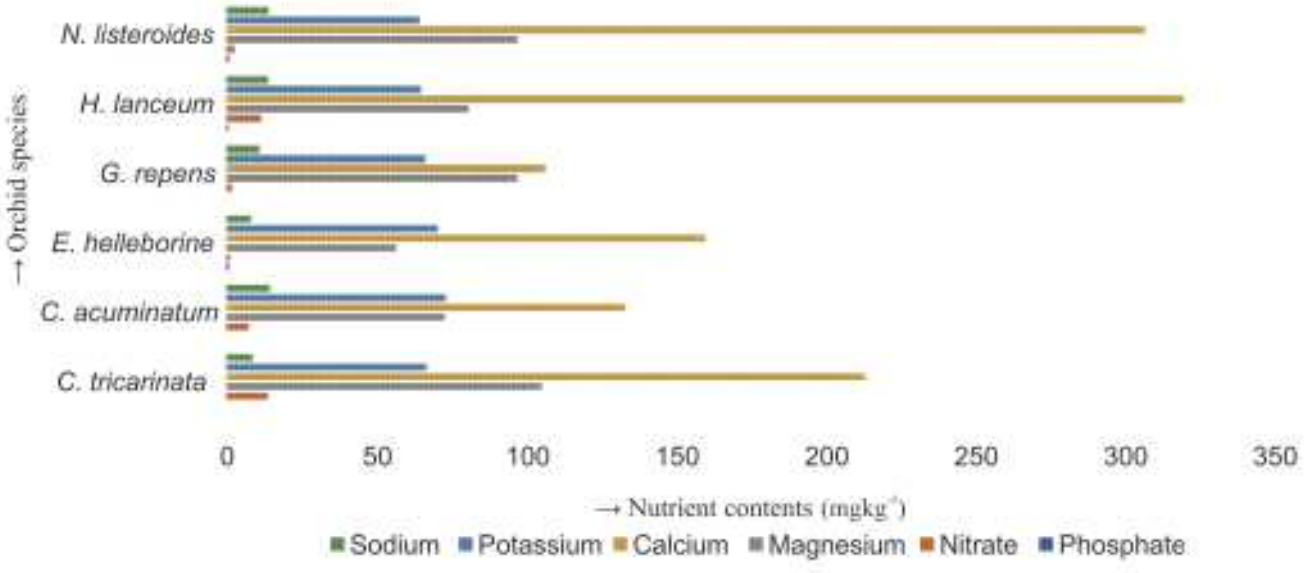

Fig. 2. Nutrient contents after analysis of soil samples of presently studied orchid species.

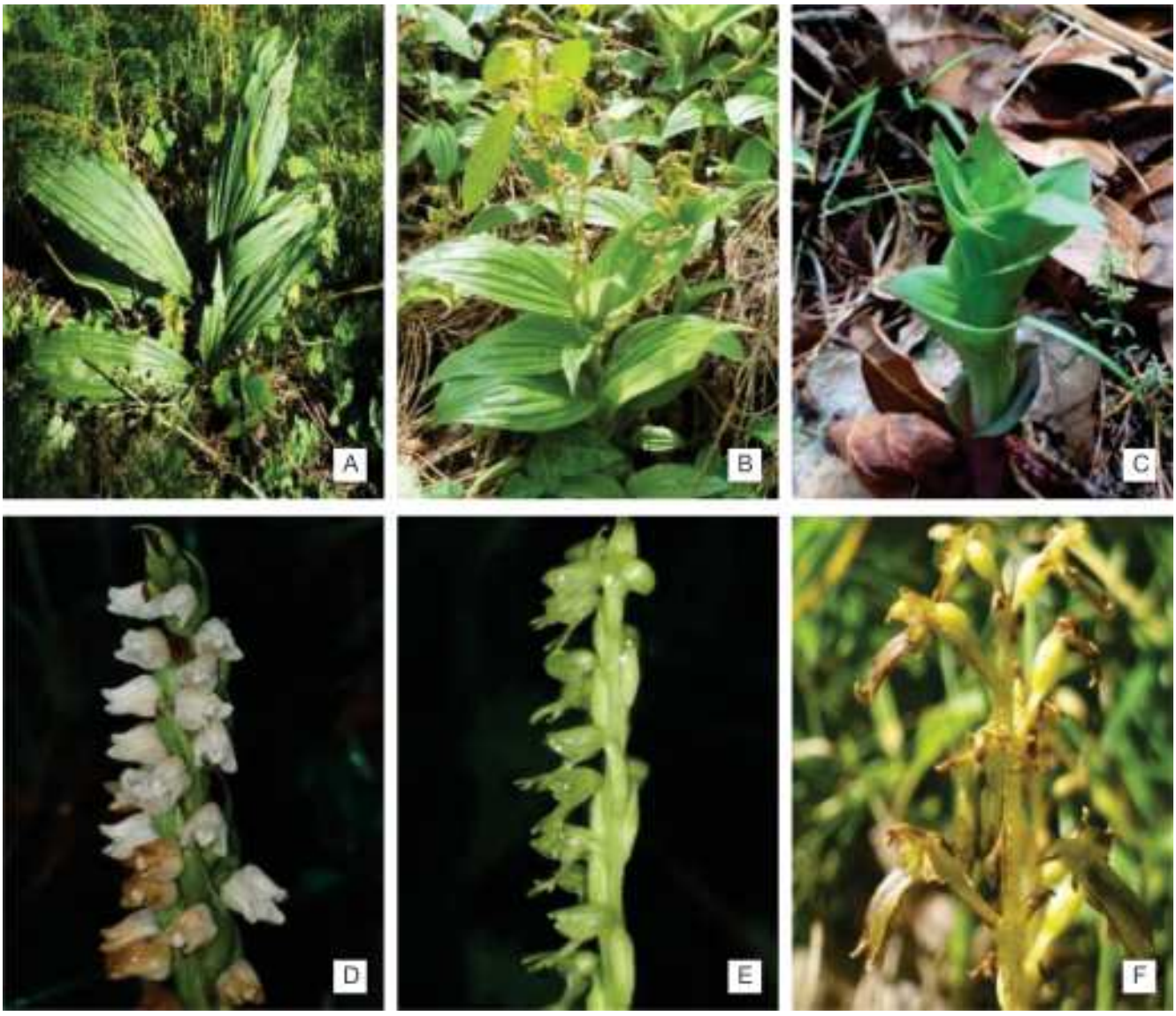

Fig. 3. A-F. Some of the orchids of Shimla hills, presently studied: A, *Calanthe tricarinata*; B, *Crepidium acuminatum*; C, *Epipactis helleborine*; D, *Goodyera repens*; E, *Herminium lanceum*; F, *Neottia listeroides*.

(Table 3). According to Poole and Seeley (1978), the vegetative growth of orchids is satisfactory at low concentrations of Potassium and is not affected by its different levels. An exogenous supply of Potassium is not a limiting factor in orchid growth; it can be mobilised (translocated) from old tissues and re-utilized to meet most of the growth requirements of new organs (Davidson, 1960). *H. lanceum* and *N. listeroides* had high Calcium, while *C. acuminatum* and *G. repens* occupied low Calcium substratum as compared to other species (Fig. 2). High concentration of Magnesium was recorded for three species, *C. tricarinata*, *G. repens*, and *N. listeroides*. Nitrate contents were very low except for *C. tricarinata* and *H. lanceum.* It was found to affect orchid distribution and directly inhibit seed germination (Figura *et al*., 2019; Ponert *et al*., 2013). Phosphate was very low or below detection limit for majority of the presently studied species. Similarly, low Phosphorus requirement for orchids was indicated earlier by a few workers (Vij *et al*., 1998; Wynd, 1933). Penningsfeld and Fast (1970, 1973) also hinted at lower requirement of orchids for Phosphorus than for Nitrogen and Potassium.

Plant tissue culture techniques have become vitally important for pursuing a wide range of fundamental and applied problems in research and development. The composition of the medium is a determining factor for successful *in vitro* asymbiotic seed germination and further growth and development of seedlings. The nutrient media for most plant tissue culture comprise five groups of ingredients: inorganic nutrients, Carbon source,

vitamins, growth regulators, and organic supplements. Standard Mitra *et al.* (1976) medium has major salts $MgSO_4.7H_2O$, KNO_3, $(NH_4)SO_4$, NaH_2PO_4, and $Ca(NO_3)_2.4H_2O$; similarly, Murashige and Skoog medium is with major salts $MgSO_4.7H_2O$, KNO_3, NH_4NO_3, KH_2PO_4, and $CaCl_2.2H_2O$. Successful plant tissue culture for a particular species depends on the choice of nutrient medium. With the help of the data obtained during the present investigation of substratum analysis, the standard nutrient media such as M (Mitra *et al.*, 1976) and MS (Murashige and Skoog, 1962) media can be modified (for successful *in vitro* asymbiotic seed germination and further growth and development of seedlings) according to the mineral or nutrient status observed for the currently studied orchid species. In both, M and MS media major salts $MgSO_4.7H_2O$ (Mg source) and KNO_3 (K source) are common with sucrose (Carbon source) (Tables 4-5). Similarly, pH in both standard media ranged from 5.6-5.8. Beside this, M medium has NaH_2PO_4 as source of Sodium and as well as Phosphorus with $Ca(NO_3)_2.4H_2O$ as Calcium source, while in MS medium KH_2PO_4 and $CaCl_2.2H_2O$ are considered as Phosphorus and Calcium source, respectively. In modified M medium, NaH_2PO_4 is considered as source of Sodium and for Phosphorus its concentration will be same like KH_2PO_4 as considered in modified MS medium with negligible concentration for *C. tricarinata, C. acuminatum,* and *G. repens,* because Phosphorus was below detection limit for these species. Both modified nutrient media have no change in the concentration of minor elements, vitamins, EDTA, and Agar. pH is also an important aspect of nutrient media and may be adjusted as per observed for the species (Table 2). Beside this, we can use different growth regulators (IAA, IBA, BAP, KN, TDZ *etc.*), Activated Charcoal, Peptone, Coconut Water, Casein Hydrolysate, Yeast Extract, Banana Extract *etc.* during their *in vitro* seed germination.

The present studies indicated that orchids of Shimla hills have a variety of habitats (shady, moisture rich to open grasslands, meadows or riverine), different soil types with slightly acidic pH, low electrical conductivity, and varied nutrient contents for different species. The data obtained through substratum analysis of six therapeutically significant and/or endangered orchids would be highly beneficial for devising nutrient formulations after modifying standard nutrient media for successful *in vitro* asymbiotic seed culture, mass multiplication, seedling development and hence for conservation of the presently studied species and other related commercially important and/or endangered Indian orchid species.

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