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Identification and Nutritional Analysis of a selected Terrestrial Orchid

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Abstract: Current study was designed with the main purpose to study terrestrial orchid species of Karnataka for their mycorrhizal association. The present investigation on the study of mycorrhizae in the selected terrestrial orchid included collection of orchid plant, rhizosphere soil samples and nutritional analysis of rhizosphere soil. The terrestrial orchid was collected from natural habitats from various locations of Kemmangundi and Sagar provinces of Karnataka State and was later investigated for mycorrhizal association. The rhizosphere soil samples along with underground parts the above plants were collected. Both physico-chemical and chemical analysis of the soil like determination of pH, electrical conductivity (EC), organic carbon, macro and micronutrients were carried out. Soil analysis in all the samples have revealed that even though soil was rich with humus and contained adequate amount of soil nutrients, phosphorus was found to be low. Soil phosphorus availability was positively related to mycorrhizal diversity probably because under low soil phosphorus availability plants and fungi are both phosphorus limited.

Keywords **-** Terrestrial Orchid, Western ghats, Rhizosphere soil, Mycorrhizal association.

I. Introduction

The term *mycorrhiza* was coined by Frank in the year 1885 for the fungus infested roots (Frank, 1885). Mycorrhizae are symbiotic associations between specialized soil fungi and plants. They are primarily responsible for nutrient transfer and are essential for one or both the partners. Most plants in natural ecosystems have mycorrhizal association when symbiotic fungi inhabit healthy tissues of the root of terrestrial plants and play an important role in their successful colonization. The orchids are mycorrhizal have been known for over 125 years by the works of Wahrlich and Janse who surveyed a large number of temperate and tropical orchid species and noted the regular occurrence of fungal colonization of the roots (He *et al.,* 1997). Many orchid products are known to have medicinal properties and are used as aphrodisiacs, treatment of sores, vermifuges (Lawler,1984).

Most terrestrial orchids are green and photosynthetic some orchids are achlorophyllous and myco-heterotrophic (Leake, 1994). All orchid species pass through a prolonged seedling stage during which they are unable to photosynthesize and depend on exogenous supply of carbohydrate by mycorrhizal fungi. These fungi are also involved in nutrient uptake especially phosphate. Orchids produce the smallest seeds by size (0.25-1.20 X 0.09-0.27mm) and weight (0.31-24µg) of any seed bearing plant (Arditti and Ghani, 2000), to compensate for this, the seeds are found in thousands and millions per one seed capsule. Orchid seeds lack metabolic and functional endosperm. Germinating seeds get their energy and nutrition from symbiotic fungus. (Burgeff, 1959). They cannot be propagated like other angiosperm seeds. In nature, orchid seeds need to be infected by suitable symbiotic mycobiont for normal growth and development (Arditti *et al.,* 1984). The advantage of such an association is that the mycorrhizal relationship increases plant access to soil resource thereby increases its tolerance to environmental stress (Rasmussen, 1995). The presence of mycorrhizal fungi in the roots of young seedlings may protect the seedling from pathogens (Clements *et al.*,1986). and a successful symbiotic relationship is established in terms of germination and seedling development (Hadley *et al.,* 1971). Unfortunately, in orchids only a few seeds (0.2 - 0.3 %) out of the millions produce germinate symbiotically in nature (Arditti *et al.*, 1984).

Bernard isolated and identified *Rhizoctonia* orchid fungi which he found could grow indefinitely on nutrient media and would form typical mycorrhizae in germination studies (Bernard 1911). Though the surveys of orchid fungi in different geographical areas have been limited, the species from Australia, Italy, Japan, and Canada appear to be similar (Currah and Zelmer, 1992; Ramsay *et al.*, 1987). The survival of orchids is nowadays severely threatened due to habitat loss, deforestation, diseases, pests, weed, encroachment, anthropogenic impacts on tropical rain forests, illegal poaching, commercial overexploitation and cultivation (Swarts and Dixon, 2009). Similar situation prevails in India where there is continued denudation of natural vegetation an alarming rate.

Orchids occur in India with Himalayas as the main home and others are scattered in the Eastern and Western India, epiphytic orchids in North Eastern India and small flowered orchids in the Western Ghats. Orchids form 9% of flora in India. Among the 924 wild species of orchids listed from the present boundaries of India, in about 132 genera, 287 species distributed over 71 genera are endemic. This indicates that 31% of orchid species are endemic to India (Jain and Mehrotra 1984). In Karnataka floristic lists including orchids have been published from time to time (Santapau and Kapadia,1966; Saldanha and Nicolson,1976; Murthy and Yoganaarasimhan,1990; Rao and Razi,1981; Yoganarasimhan *et al.* 1981), and there are more epiphytic orchids than terrestrial ones. Terrestrial orchids (19 genera and 62 species) occur mainly in the Western Ghats region of Karnataka. Though taxonomic study of orchids is well established the microbiology of orchids is little known (Kottaimuthu *et al.*,1998).

Furthermore, review of literature revealed that there are only a few reports on studies on mycorrhizal associations in orchids especially from India. (Vij *et al.*, 1988; Katiyar *et al.*,1986; Siddique and Raghuvanshi, 1993; Senthilkumar *et al.*, 2001; Saha and Rao 2006; Sharma *et al.*, 2007; Aggarwal *et al.*, 2010) Hence, the present work we aimed to study terrestrial orchid species of Karnataka for their mycorrhizal association.

II. Materials and Methods

The materials and methods used in the present investigation on the study of mycorrhizae in terrestrial orchids included collection of orchid plants and rhizosphere soil samples and nutritional analysis of rhizosphere soil of selected ground orchid.

Collection of Orchid Plants and Rhizosphere soil samples

The plant materials were collected from natural habitats of Western Ghats and from different ecological niches so that diversity in root system and mycorrhizal association was reflected therein (Plate 1 and Figure 1).

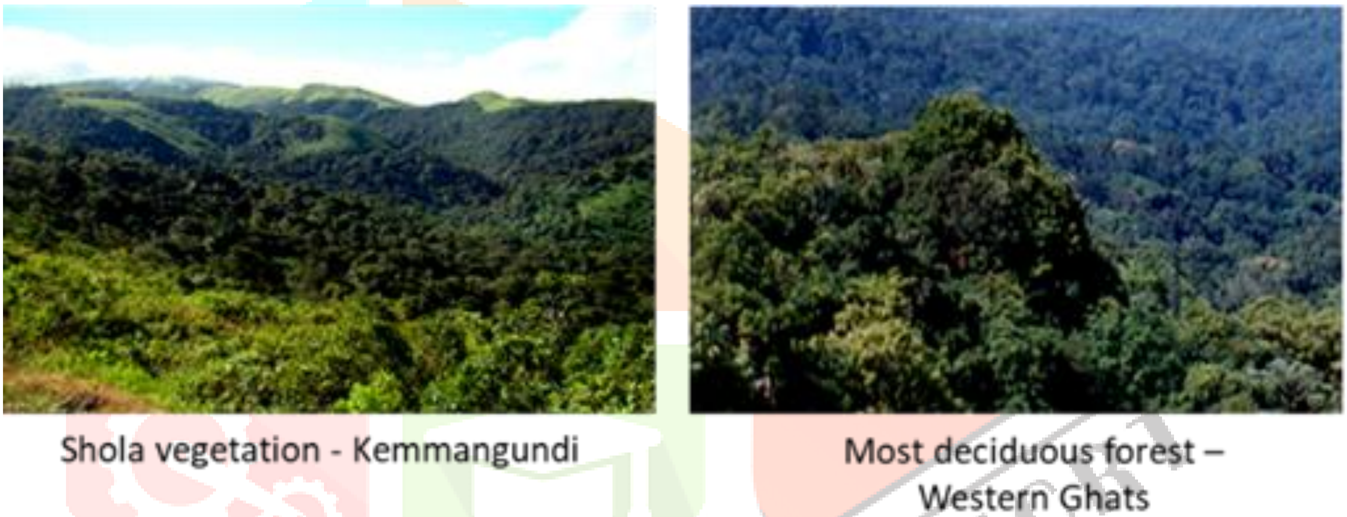


Plate 1

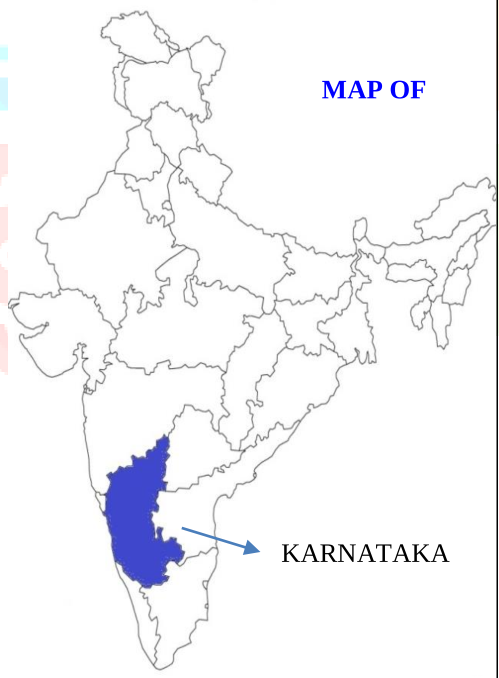
Orchids have delicate root system and therefore the plant was carefully dug to avoid damage to the root system. The underground part (root/tuber/pseudobulb) and rhizosphere soil samples were collected in clean polythene bags and labeled (Plate 2). Orchid plant specimens were preserved and identified using taxonomic keys published in various Floras (Abraham, 1981). Wet method of herbaria preparation by Saldanha and Nicolson was followed (Saldanha and Nicolson, 1976).

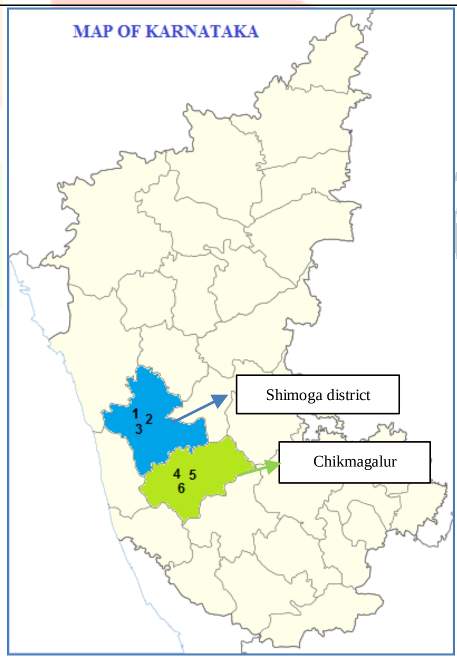
Habit of *Nervilia crociformis*

Inflorescence of *Nervilia crociformis*

Tuber of *Nervilia crociformis*

Plate 2





Collection site - Shivmoga	Orchid Plant
1. Sagar	1. *Nervilia crociformis*
2. Linganmakki reservoir	
3. Kogar	

Figure 1: Map of Karnataka showing collection sites

The terrestrial orchid and their rhizosphere soil samples were collected from the moist deciduous forests and from the adjoining grasslands close to shola forests of Kemmangundi, Chikmagalur district, Sagar and adjoining areas of Shimoga district, Karnataka. Kemmangundi is a hill station located at Tarikere a picturesque hill station situated at a height of 1434m above sea level. The hill station is ringed by Baba Budan Giri Range with cascades, mountain streams and lush vegetation. It is located at 13°33' N and 75°45'. It is called "Ooty of Karnataka" because of its cool climate during summer. Sagar is a town located at $14^0$17'N and $75^0$03'E. The surrounding places like Jog falls, Linganmakki, Kogar have moist deciduous to dry deciduous forests supporting a large number of ground orchids.

Nutritional Analysis of Rhizosphere Soil of Selected Ground Orchid

Rhizosphere soil analysis was carried out to study the soil quality and the prevalence of orchid mycorrhiza in the natural habitats. Both physico-chemical and chemical analysis like determination of pH (soil reaction), conductivity (EC), organic carbon, available macro and micronutrients were carried out using standard procedures as outlined by Tandon et al (Tandon *et al.* 1993).

pH

pH is a measure of hydrogen or hydroxyl ion activity of soil-water system. It indicates whether soils are acidic, alkaline or neutral. Standard buffer solutions of pH 4.0, 7.0 and 9.2 in pure water was prepared. Saturated soil paste was made using 100g of soil sample in 500mL. distilled water which was added at 1-2 min intervals. The beaker was allowed to stand for an hour till soil paste glistened. The pH meter was adjusted for suitable temperature and calibrated with two buffers, one acid and the other alkaline or neutral and pH for each sample was determined.

Electrical conductivity (EC)

Ions like metals allow electric current to pass through them. Hence EC of the soil-water system rises with increasing content of soluble salts in the soil. Thus, measurement of EC will give the concentration of soluble salts in the soil at any particular temperature. Standard 0.01N potassium chloride was used to calibrate the conductivity meter. The soil sample was measured in known volume of distilled water and agitated in a shaker. It was allowed to stand till clear supernatant liquid was obtained. The conductivity meter was calibrated and EC was measured in the supernatant liquid.

Organic carbon

Soil organic matter (SOM) is the seat of nitrogen in the soil and its determination is carried out as an index of nitrogen availability. Organic carbon was estimated using Colorimetric method (Datta *et al.*, 1962). The organic matter is oxidized with chromic acid (potassium dichromate and sulphuric acid). The intensity of the green colour of chromic acid obtained due to reduction was measured colorimetrically. This intensity is directly proportional to the amount of organic carbon present in the soil.

Phosphorus

In soil phosphorus exists in the form of various types of orthophosphates. A small fraction of these is available to the plants at a given time. Available phosphorus content of the soil consists mainly of calcium, aluminum and iron-phosphorus fractions. In neutral and alkaline soils particularly calcium-phosphorus fractions are dominant. Bray and Kurtz P-1 method was used for alkaline soils (Bray and Kurtz, 1945). Phosphorus extracted in the above methods was determined using colorimeter by measuring the colour intensity at 660nm wavelength. Potassium in soil exists as water soluble, exchangeable, non-exchangeable (fixed) and lattice potassium. Ammonium acetate method was used as ammonium ions are close in size to potassium and replace the latter (Hanway and Heidel, 1952) During estimation acetate burns cleanly and does not leave any residue on burner on flame photometer.

\Micronutrients (Zinc, Iron, Copper and Manganese)

Neutral ammonium acetate, chelating agents like EDTA (Ethylene diamine tetra acetic acid) DTPA (Diethylene triamine penta acetic acid) were used for extractants like Zn, Mn, Fe. These chelating agents combine with free metal ions in solution to form soluble complexes. Colorimetric method was used for estimation of available micronutrients.

III. RESULTS AND DISCUSSION

The terrestrial orchid, selected for mycorrhizal study, were species of *Nervilia crociformis* (collected from Sagar, Shimoga district, Karnataka). They were identified using taxonomic keys published in various Floras like Ananda Rao (Ananda Rao, 1988).

Collection of Terrestrial Orchids:

Nervilia Comm. ex Gaud. is an old world genus of about 100 species with small underground tubers, single leaf and an inflorescence of one to many flowers. Leaf and flowers appear at different times. India is known to have around 16 species, of which 6 are found in South India with 4 species recorded from Karnataka.

***Nervilia crociformis* (Zoll. ex Mor.) Seidenf.**

Hystaranthous tuberous terrestrial species, leafless when in flower. Leaf adpressed to the ground, orbicular, cordate, petiolate, plicate, hairy above and glabrous beneath. Flowers resupinate, solitary, white, terminal, sepals spreading, ovate; petals smaller, narrow, lanceolate, lip long, 3-lobed, purple with a median yellowish callus; column curved. It is widely distributed in Gujarat, Rajasthan, Kerala, Tamil Nadu; Sikkim (La Chung Valley); Mysore, Kodagu, Hassan, Shimoga, Uttara Kannada in Karnataka. Flower blooms and bears fruiting in March to April, flowers appear with the first showers and last only for a day. (Plate 1)

Nutritional Analysis of Rhizosphere of Selected Orchids:

The term rhizosphere refers to the soil immediately surrounding the root system. The rhizosphere region is a highly favorable unique subterranean habitat for proliferation and metabolism of numerous types of microorganisms (Alexander, 1977). It is a seat of intense plant and soil microbial interaction. Despite several studies have reported association with bacteria in orchids from different locations, particularly in epiphytic orchids (Tsavkelova *et al.*, 2004), the study of association with partially mycoheterotrophic terrestrial orchids is limited (Girija *et al.*, 2018, Tsavkelova *et al.*, 2007). Certainly, carbon is the main nutritional compound required by the embryo during the protocorm stage, but when the plantlet stage starts, the nutritional demand requires other nutrients to start growth and development (Cameron *et al.*, 2008, Ogura *et al.*, 2009). Here, plant growth-promoting bacteria can have a direct role by the production of auxin, phosphate solubilization or providing nutrients necessary to support growth and autotrophy (Afzal *et al.*, 2019). Inside orchid roots, bacteria may interact with fungi and orchid root cells to maintain the beneficial effect of the symbiosis, directly by production of plant growth-promoting metabolites or indirectly by the inhibition of fungal growth that can be harmful to the plant (Kuga *et al.*, 2014; Tsavkelova *et al.*, 2016). Such processes have been widely reported in endophytic and rhizospheric bacteria isolated from several plants; however, association of partially mycoheterotrophic terrestrial orchids from the southern Andes has not yet been reported. The microorganisms are mainly soil inhabiting fungi and bacteria. Some of the soil fungi form mycorrhizal association with the neighboring plants. Soil nutrients along with root exudates play an important role in their colonization. Rhizosphere soil analysis helps in the evaluation of soil fertility. Hence rhizosphere soil of six terrestrial orchids were analyzed for the physico-chemical and chemical parameters. The results of the analysis were as depicted in Table 1.

Table 1: Rhizosphere Soil analysis

	pH	EC (ds)	Organic Carbon (%)	Phosphorus (Kg/ac.)	Potassium (Kg/ac.)	Zinc (ppm)	Iron (ppm)	Copper (ppm)	Manganese (ppm)
Normal limits	6.3-8.5	<1	0.5-0.75	9-22	60-120	1	2	0.2	2
Nervilia crociformis	5.9	0.08	2.11 (H)	5 (L)	152 (H)	11.7 (S)	37 (S)	1.1 (S)	47.3 (S)

H-High; L-Low; S-Saturated

pH

Rhizosphere effects can induce an increase or decrease in the decomposition of soil organic carbon (SOC), which is frequently referred to as a positive or negative rhizosphere priming effect (RPE). These rhizosphere effects include root release of organic C and N substances, depletion of nutrients and water, or root-induced chemical changes such as soil pH. Root-mediated changes in rhizosphere pH are a well-documented interaction between soil and the root interface (Hinsinger *et al.*, 2003). Hence we studied the analysis of the soil quality showed the variations from one collection site to the other. Soil pH was observed to be acidic to almost neutral. The reason for the increased levels of acidity is that as water moves through the soil, there is a tendency for bases to be leached out and replaced by hydrogen ions thus constant leaching leads to formation of acidic soils. Such changes affect the chemical properties of the soil, altering the solubility of certain substances. Under acid conditions, many substances become more soluble and Calcium and Phosphorus compounds may be leached out resulting in nutrient deficiencies.

Electrical conductivity (EC)

Other than anchoring the plant and absorbing water and nutrients, roots also release organic and inorganic substances to alter the rhizosphere. The tendency to secrete such substances and the type of substances released are affected by a number of factors such as plant species and nutrient concentration (*Pinton et al.*, 2007). As the plant releases exudates to the rhizosphere through anion channels, the H^+-ATPase on cell membrane discharges a proton to balance the electrical charge, causing the pH of the rhizosphere to decline. In our study electrical conductivity (EC) is in the normal limits in all the six samples studied. Ions allow electric current to pass through them and hence EC of soil-water system rises with increasing content of soluble salts in the soil. Measurement of EC gives the concentration of soluble salts in the soil at a particular temperature. The rhizosphere soil samples of *Calanthe sylvatica, Malaxis rheedei* and *Satyrium nepalense* collected from Kemmangundi showed low EC values indicating low availability of soluble salts.

Organic carbon

The rhizosphere is a biologically active zone in soil where complex interactions occur among plant roots, soil particles and microbes. Investigations on the rhizosphere help to gain a better understanding on a variety of processes such as nutrient cycling, ecosystem functioning, carbon dynamics, and to evaluate the impacts of anthropogenic activities on agricultural and natural ecosystems. An important rhizosphere process is rhizodeposition, that involves the transfer of carbon from plant roots to soil in the form of water-soluble exudates (low molecular weight molecules such as organic acids, amino acids and sugars), secretions of higher molecular weight substances, lysates from cells after autolysis, gases such as ethylene, carbon dioxide, and hydrogen cyanide (Grayston *et al.*, 1996). Through rhizodeposition, plants supply soil microbes with readily available organic carbon, which increases microbial biomass around roots and affects activity and composition of microbial communities. On the other hand, microbes mobilize nutrients, primarily nitrogen, and render them available to plants, as described by the "microbial loop" model (Bonkowski, 2004).

Rhizosphere nutrient flow also plays an important role in organic carbon mobilization and immobilization processes with important con-sequences on carbon dioxide fluxes involved in the greenhouse effect (Cheng and Johnson, 1998). In our study we found that the soil was rich with organic carbon in all the samples due to high plant litter decomposition activity by soil microorganisms. Organic matter provides a reserve of plant nutrients like soil Nitrogen, Phosphorus and Sulphur which exists in organic form and are made available to growing plants by its decomposition.

Phosphorus

Phosphorus is the second most important mineral nutrient for plant growth after Nitrogen. Although total phosphate is abundant in many soils, it is largely unavailable for root uptake because, about 20–80% of soil phosphorus occurs in organic forms (*Richardson et al.*, 2009). Soil phosphorus easily forms insoluble complexes with cations, and/or incorporated into organic matter by microbes (Vance *et al.*, 2003). Even where phosphorus is applied as fertilizer, more than 80% becomes unavailable for plant uptake through adsorption, precipitation, or microbial immobilization (Holford, 1997; Mózner *et al.*, 2012). In soil, phosphorus exists in the form of orthophosphates. A very small fraction of these is available to plants at a given time. Though the soil was rich with humus, phosphate content was found to be low in all the samples. Since phosphorus was scanty in the soil, which prompted the orchid plants to have mycorrhizal association with native fungal species.

Potassium

Potassium (K) is the third major essential macronutrient for plant growth. The concentrations of soluble potassium in the soil are usually very low and more than 90% of potassium in the soil exists in the form of insoluble rocks and silicate minerals. Available potassium exists in the form of water soluble, exchangeable, non-exchangeable (fixed) and lattice-K. The first two forms constitute a small part, considered to be easily available to plants (Richardson, 2001).

Micronutrients (Zinc, Iron, Copper and Manganese)

The ability for plants to form symbiotic associations with mycorrhizae occurred early in the evolution of plants (~450 million years ago) compared to legumes (~60 million years ago), which is likely why mycorrhizae are now ubiquitous throughout the plant kingdom. Although parasitic and neutral relations exist, a majority of these associations are beneficial both to the host plant and the colonizing fungi. Mycorrhiza assist plants in obtaining water, phosphorus and other micronutrients (e.g., Zn and Cu) from the soil and in return receive sustenance (carbon) from the plant. The soil samples analyzed contained adequate to high amount of available potassium. Available micronutrients (Zn, Cu, Fe and Mn) which are essential and required in minor quantities for plant growth was found to be scanty in all the six samples (Maseko *et al.*, 2013).

IV. CONCLUSION

The terrestrial orchid identified as *Nervilia crociformis*, was collected from natural habitats from various locations of Kemmangundi and Sagar provinces of Karnataka State, and were investigated for mycorrhizal association. The analysis of the rhizosphere soil in all the samples revealed that even though soil was rich with humus and contained adequate amount of soil nutrients, phosphorus was found to be low. Since phosphorus was scanty in the soil, it prompted the orchid plants to have mycorrhizal association with native fungal species.

Soil phosphorus availability was positively related to mycorrhizal diversity probably because under low soil phosphorus availability plants and fungi are both phosphorus limited. According to the results it can be concluded that the type of mycorrhiza in the soil and climatic factors of the region are more effective in orchid species distribution than the soil characteristics.

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