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A New Record of Endemic and Endangered Mycorrhizal Plant: Ceropegia Media (Huber) Ansari From India

Vishal R. Kamble[1], Ruchira R. Sutar[2]
[1-2]Mycorrhizal Research Laboratory PG Department of Botany,Bhavan's College Andheri (West), Mumbai (MS) 400058 India Accredited 'A' by NAAC (2014-2015) [1]Corresponding Author

Abstract: *Present paper deals with, detailed assessment of mycorrhization established by Arbuscular mycorrhizal fungi (AMF) with Ceropegia media (Huber) Ansari, i.e. species considered as endemic and endangered to one of the world's biodiversity hotspots region in Western Ghats of Maharashtra, India. Moreover, the first endemic genus was studied for the first time in respect of AMF root colonization from India. Microscopic observations of cleared and stained roots showed 55.77% AMF colonization. Eight species of AMF were identified in the rhizosphere soil viz., Acaulospora scrobiculata, A. sporocarpa; Ambispora callosa, A. reticulata; Gigaspora decipiens, Glomus caledonium, G. Etunicatum and G. microaggregatum. Based on spore density and relative abundance, two species were dominant (S ≥ 40 spores 100 g^{-1} soil, RA > 6%) viz, Ambispora reticulata (40 spores, 20.10%) and Glomus microaggregatum. (82 spores, 41.20%). Based on in depth study C. media is proposed as mycorrhizal plant.*

Keywords: *Arbuscular mycorrhizal fungi (AMF), endemic and endangered plant, Ceropegia media (Huber) Ansari*

I. Introduction

Out of 58 Indian species of *Ceropegia* (Apocynaceae, Ceropegieae, Stapeliinae) 35 species and one variety are endemic to Western Ghats most of which are critically endangered, endangered or vulnerable as per the IUCN categories and are hence listed in the *Red Data Book of Indian Plants* **[1]**. Out of these, 17 species and one variety are strictly endemic to Maharashtra, *Ceropegia media* (Huber) Ansari is one of them (**Figure 1**). *C. media* is a slender, tuberous, twining perennial herb, having narrowed to broad leaves and with many flowered cymose inflorescence. It grow at higher elevations (altitudes from 500-1500 m) of Sahyadri ranges in Western Ghats of Maharashtra, India especially area adjoining to Ahmednagar (Kalsubai), Pune (Purandhar, Bhimashankar, Khandala, Sinhagad, Ambavane, Varandh), Ratnagiri (Marleshwar), Sangli (Ghotane) and Satara (Thoseghar plateau, Kas plateau). *C. media* was earlier reported from Pune and Satara districts only. In 1998 it has been reported from Kalsubai hill of Ahmednagar district **[2]**. It is having flowering period during July – September whereas fruiting during August - October.

Figure 1. Endemic and endangered plant *Ceropegia media* (Huber) Ansari:
a. Study area Visapur Fort**; b.** Habitat; **c.** Flower

Like the other species of *Ceropegia*, *C. media* is under threat, owing to either destructive collection or habitat degradation caused by anthropogenic activities. It is not only genetically depleted but also is scarcely available and hence considered as endangered. The tubers are considered edible and delicate flowers have ornamental value. However, it is difficult to maintain *C. media* in gardens which needs *in-situ* conservation **[3]**.

Several active plant protection and conservation techniques are being applied to multiply plant species *ex situ* and to maintain natural populations of these species. According to **Holl and Hayes [4] "**introducing rare plants to new sites for conservation to offset effects of habitat destruction requires detailed knowledge of habitat requirements, plant demography, and management needs". Unfortunately in conservation of such rare plant species, the diversity and dynamics of macro and micro habitats associated with them is not taken in to consideration. With increasing pressure and an anthropogenic activity, the composition of the forest is changing day by day, thereby directly affecting the diversity and dynamics of macro and micro habitats. Many organisms and species may become extinct before their potential is realized **[5]**. Although the soil microbes associated with such threatened species which are key links in the soil nutrient and energy cycling, the vast world of such soil microbes has remained unfocused on agenda of biodiversity conservation. Soil microorganisms, especially Arbuscular Mycorrhizal Fungi (AMF), are considered to be crucial for proper plant performance **[6], [7].** Therefore monitoring of soil and the selection of appropriate microbial strains to inoculate the plants to be protected could be of particular value. AMF are considered useful in the development of effective methods for the maintenance and propagation of threatened plant species and may significantly improve the success of plant conservation actions **[8], [7], [9].** Hence, it is urgently needed to conduct explorations on such genuine AMF species associated with rare endangered and threatened plants so that technology can receive some valuable inputs about utilization of potential of these AMF species in conservation plan to be set. With this perspective, in present paper an attempt has made to understand mycorrhizal colonization pattern and AMF species associated with endemic and endangered plant *C. media* for first time.

II. Materials and methods

2.1 Site description:

During present investigation this species was observed in a population of about 15 – 20 plants for first time at Visapur fort which is a new location for distribution pattern of *C. media*. The study area is located in Pune district at elevation of 1084 meters above sea level and coordinates 18° 43'21" North and 73° 29' 24" East **[Figure 1].** This area is exposed to extensive anthropogenic activities leading to threat for habitat destruction of *C. media*. The plant grows around the bushes, shrubs and in grasses on the steeping slopes of hills or along the forest borders. Extensive field visits were carried out during July – October for better understanding of plant species from taxonomical perspective. Plants identification and nomenclature is that of **Jagtap and Das-Das. [10]**. Authentically identified plant specimens were dry preserved and deposited in departmental herbarium.

2.2 Soil sampling:

Sampling patches were selected haphazardly with precaution, that there should not less than 5-10 saplings. Total three plants were sampled for soil collection. However, only two plant specimens were sampled for root screening study purpose, so that these plants should not be damaged or destroyed further. The litter and stony particles were scrapped off from upper soil surface of *C. media*. The was plants were removed from the natural habitats carefully with help of digger. The plants along with the rhizosphere soil samples and roots were collected in different collection bags. The collection bags were closed air tight to maintain the moisture and freshness of the plant, Labelling of bags were done which includes - the date, time, location and transported to laboratory and immediately refrigerated at 4°C subsequent to arrival. The roots were processed immediately. All the rhizosphere soil samples from triplicate were homogenized prior to remove coarse roots segments, stones and adhered particles through sieving procedure (2 mm mesh size). Subsamples of triplicate soil were air dried and used for estimation of physico-chemical properties.

2.3 Physicochemical parameters of soil:

Soil texture was estimated gravimetrically by hydrometer method **[11]**. As per the procedures for soil pH analysis **[12]**, sample was analysed on 1:2.5, soil : water suspension. Organic carbon was analyzed by WB rapid titration method **[13]** using 1N potassium dichromate and back titrated with 0.5N ferrous ammonium sulphate solution. Carbonate was estimated by Piper's rapid titration method **[14]** and available Olsen's phosphorus in soils was determined by extraction with 0.5M sodium bicarbonate for 30 min **[15]**.

2.4 AMF colonization in Roots:

To determine the colonization percentage, root samples were washed under running tap water, and cut in segmments 1cm in length and stained for rapid mycorrhizal association following the method of **Phillips and**

Hayman [16]. The root segments were treated with 10% KOH solution and autoclaved at 15 lbs for 20 min and treated with 1% HCL followed by staining with 0.5% lactophenol cotton blue for 24 hour. After 24 hour the root samples were ready for screening under microscope. Assessment of root colonization was performed on each stained root piece by putting it on the slide containing mounting medium lactophenol and covering with a cover slip followed by microscopy. Fifty stained root segments were assessed for colonization percentage using the intercept method **[17]** under a Olympus compound microscope. A root segment was considered for counting as colonized by AMF when any mycorrhizal component such as hyphae, vesicles or arbuscles was observed. All the three AMF components were interpreted for occurrence intensity *viz., poor* (1-25%), *moderate* (25-50%), *good* (50-75%) and *excellent* (≥75%) which was denoted as *P*, *M*, *G* and *E* respectively. Based on microscopic observations for randomly selected 50 root segments pattern of AMF colonization for *C. media* was determined. Any other special structures and other fungal endophytes present in root were also recorded.

2.5 AMF spore extraction:

Spores were extracted from the100g of rhizosphere soil with the help of different size of sieves ranging from 25-250 µ by using sieving and decanting technique **[18]**. Total spore numbers of AMF in the soil sample were estimated following **Gaur and Adholeya [19].** The spore densities were expressed as the number of spores per 100g of soil. The spores retained on each sieve were transferred to filter paper and subsequently picked up with the help of pasture pipette or needle under dissecting microscope for assessment. All spores were mounted in a polyvinyl-lactoglycerol (PVLG) and PVLG solution mixed with Melzer's reagent 1:1 (v/v) ratio **[20]**. All the spores were examined under stereomicroscope (Olympus 003421) at 10X, 40X and 100X magnifications. Only spores that appeared to be healthy were recorded and counted. Photomicrographs were taken with the help of Canon IXUS 155 digital Camera.

2. AMF species identification:

Taxonomic placements of AMF spores and sporocarps up to species level was based on spore size, colour, wall layers and hyphal attachments after comparison with type or authenticated specimens. The identification is purely based on the synaptic keys **[21], [22], [23]** and also after consultation with descriptions of AMF species provided by International Culture Collection of Vesicular and Arbuscular Endomycorrhizal Fungi [http:// invam.caf.wvu.edu/Myc_Info/Taxonomy/species.htm]. The species codes were followed after **Schenk and Perez [24]**. Voucher slide specimens were assigned accession codes '*BCA:MH*$_{RRS}$n' [where, *BCA:MH is Bhavan's College Andheri: Mycological Herbarium;* $_{RRS}$ *initials of second Author and n is number assigned*'] and preserved in Mycorrhizal Research Laboratory of Department.

Spore density (S) was considered as the number of spores in 100 g soil. Relative abundance (RA) was defined as the percentage of spore numbers of a species divided by the total spores observed **[25]**. The dominant AMF species was determined according to relative abundance (RA > 6%) and spore density (S ≥ 40 spores). Statistical data processing for percentage colonization in roots, spore density and relative abundance of AMF species was performed for standard errors of means by using Microsoft excel 2007.

III. Results and Discussion

3.1 Physicochemical parameters of soil:

Soil requirement for any plant and associated microbes varies from species to species and hence physicochemical properties of soil should be taken into consideration. It helps to understand optimum requirements of microhabitats in addition to plant species for sustaining under natural conditions. Physicochemical properties of soil are key factors in conservation strategies for endemic and endangered plant species. Physicochemical properties of the rhizosphere soil of *C. media* are presented in **Table 1.**

The soil had alkaline pH 7.4; organic carbon 0.84%, organic matter 1.44%, carbonate content 7.32 mg.kg^{-1}, Olsen's Phosphorus content 12.1 mg.kg^{-1}. In general, soil is slightly red, coarse gravel in texture, slightly alkaline in reaction; low in organic matter, carbonate content and available phosphorus level.

Table 1: Physicochemical properties of soil for endemic and endangered plant *C. media*

Sr. No.	Parameters	Status
1.	Colour	Slightly red
2.	Soil texture	Coarse gravel
3.	pH	7.4 ± 0.01
4.	Organic Carbon	0.84 %
5.	Organic Matter	1.44 %
6.	Carbonate	7.32 ± 0.04 mg.kg^{-1}
7.	Phosphorus	12.1 ± 0.02mg.kg^{-1}

(±) Standard error of mean

3.2 AMF colonization in roots of *C. media*:

In the root segments of *C. media,* AMF hyphae (*H*) had established distinctive structures like hyphal network (*hn*), hyphal coiling (*hc*) and vesiclular primordial hypha (*vph*). Present work endow with confirmation that, the root segments were exhibiting good vesicular colonization (*V*) (66.66%), and an excellent hyphal colonization (*H*) (98.60%) **(Figure 2 a-e)** but sparingly formed arbuscles indicated poor arbuscular colonization (6.66%) (**Table 2**). Thus on the basis of microscopic observations made on cleared and stained root segments of *C. media* exhibited 55.77% AMF colonization. It was encountered with vesicular-arbuscular-hyphal type (*VAH*) pattern of root colonization. Hence we are proposing *C. media* as a mycorrhizal in status, which makes a first report of mycorrhizal endemic and endangered plant under genus *Ceropegia* from India. In addition to common AMF components, presence of other fungal endophytes along with mycorrhizal colonization was reported. Presence of dark septate hypha/e (*dsh*) and sclerotia (*sc*) in the roots of *C. media* made a diagnosis of other fungal endophytes occurrence (**Figure 2 f-g)**.

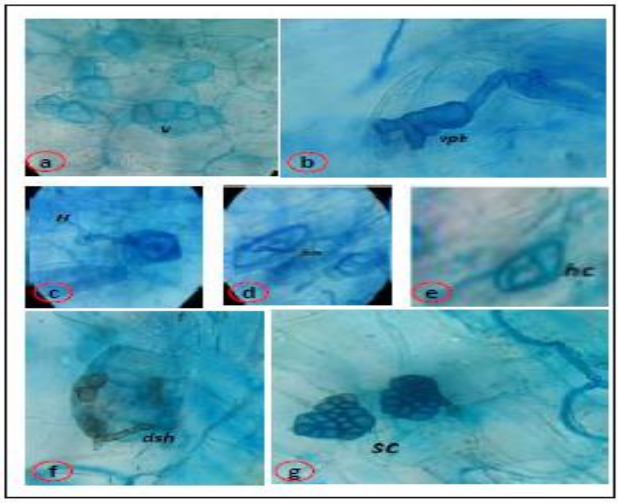

Figure 2. a. AMF colonization in roots of endemic and endangered plant *C. media*: a. vesicular colonization (*V*); **b.** vesiclular primordial hypha (*vph*); **c.** hyphae (*H*); **d.** hyphal network (*hn*); **e**. hyphal coiling (*hc*); **f.** dark septate hypha of other fungal endophytes (*dsh*) and **g.** sclerotia (*sc*) of other fungal endophytes

Table 2: Status of AMF colonization in roots of endemic and endangered plant *C. media*

Particulars	***AMF colonization in roots***		
AMF components	***Vesicles***	***Arbuscules***	***Hyphae***
Occurrence intensity (O.I.)	*66.82 ± 1.6 %*	*6.67 ± 0.8 %*	*98.60 ± 2.2%*
Interpretation on O.I.	*good[g]*	*poor[p]*	*Excellent[e]*
Remark on colonizing structures of mycorrhiza	*Formation of hyphal network, hyphal coiling & vesiclular primordial hypha*		
Remark on other fungal endophytes	*Presence of dark septate hypha/e (dsh) & sclerotia (sc)*		
Percentage root colonization	*57.36 ± 1.5 %*		
Pattern of Mycorrhizal colonization	*VAH*		

(±) Standard error of mean; (p) 1-25%, (g) 50-75%, (e) ≥75%; VAH: vesicular-arbuscular-hyphal type

3.2 AMF species identification:

Eight species of AMF were identified in the rhizosphere soil of *C media* from field scattered over five genera *viz.*, *Acaulospora, Ambispora, Gigaspora* and *Glomus*. Amongst the five genera, *Acaulospora* represented two species (25%) *viz., A.. scrobiculata* & *A. sporocarpa*; *Ambispora* (25%) *viz., A. callosa*, & *A. reticulata; Gigaspora* (12.5%) *i..e. G. decipiens* and *Glomus* (37.5%) *viz., G. caledonium, G. etunicatum* & *G. microaggregatum.* . Based on spore density and relative abundance, two species were dominant (S ≥ 40 spores 100 g^{-1} soil, RA > 6%) *viz, Ambispora reticulata* (40 spores, 20.10%) and *Glomus microaggregatum.* (82 spores, 41.20%) (**Table 3**). The morphological characteristics of all eight species of AMF are illustrated in Fig. 3.

Table 3 Identified AMF with their spore density (S) and relative abundance (RA) in endemic and endangered plant *C. media* rhizosphere (dominant species are in bold).

Code	**Species**	**S**	**RA**
BCA_{RRS}02 & 03	*Acaulospora scrobiculata* Trappe	28	14.070
BCA_{RRS}06	*Acaulospora sporocarpa* Berch**.**	06	3.015
BCA_{RRS}12	*Ambispora callosa* (Sieverd.) Walker, Vestberg & Schüssler	13	6.532
BCA_{RRS}14 & 15	***Ambispora reticulata* Oehl & Sieverd**	**40**	**20.100**

BCA$_{RRS}$16 & 17	*Gigaspora decipiens* (Hall & Abbot), Bentivenga & Morton	03	1.507
BCA$_{RRS}$22	*Glomus caledonium* (Nicol. & Gerd.) Trappe & Gerd.	02	1.005
BCA$_{RRS}$23	*Glomus etunicatum* Becker & Gerd.	25	12.562
BCA$_{RRS}$26 &27	***Glomus microaggregatum* Koske, Gemma & Olexia**	**82**	**41.206**
Total: AMF 8 species		**199**	**100**

It has well established in last two decades that below-ground diversity of AMF and their interactions works as major factor contributing to the maintenance of plant biodiversity and to ecosystem functions like productivity and variability **[26], [27], [7], [28]**. The status of AMF with rare, endemic and endangered plants at global scale revealed that, from Austria **[9],** China **[29]**, Ethiopia **[30],** India **[31], [32];** Oregon state US **[33]** and Southeast Florida US **[8]** etc. had contributed significant information**.** As far as concern with India endangered Indian plant species *viz*. *Leptadenia reticulate* (Asclepiadaceae), *Mitragyna parvifolia* (Rubiaceae), *Withania coagulans* (Solanacaea) **[32]** and *Moringa concanensis* (Moringaceae) **[31]** were documented. Survey of literature strongly admired, the application of AMF consortia, to support plant growth can be effective in conservation programmes of rare, endemic and endangered taxa **[33], [34],** [35], [36], [37] as well as for the protection of threatened taxa [38], [39], [40].

In comparison with global scenario of AMF status and their efficacy on endangered plant species *viz*. *Leptadenia reticulata*, *Mitragyna parvifolia*, *Withania coagulans* **[32]** and *Moringa concanensis* **[31]** were only documented so far from India. In present report, we recognized, to the best of our knowledge for the first time, the mycorrhizal status of *C. media*, i.e. species considered as endemic and endangered to one of the world's biodiversity hotspots region in Western Ghats region of Maharashtra, India. Moreover, the first endemic genus was studied for the first time in respect of AMF root colonization from India. These studies provide important basic information about interaction between *C. media* and associated eight AMF species. Thus it may facilitatthe potential use of AMF in endeavors aimed at *C. media* conservation.

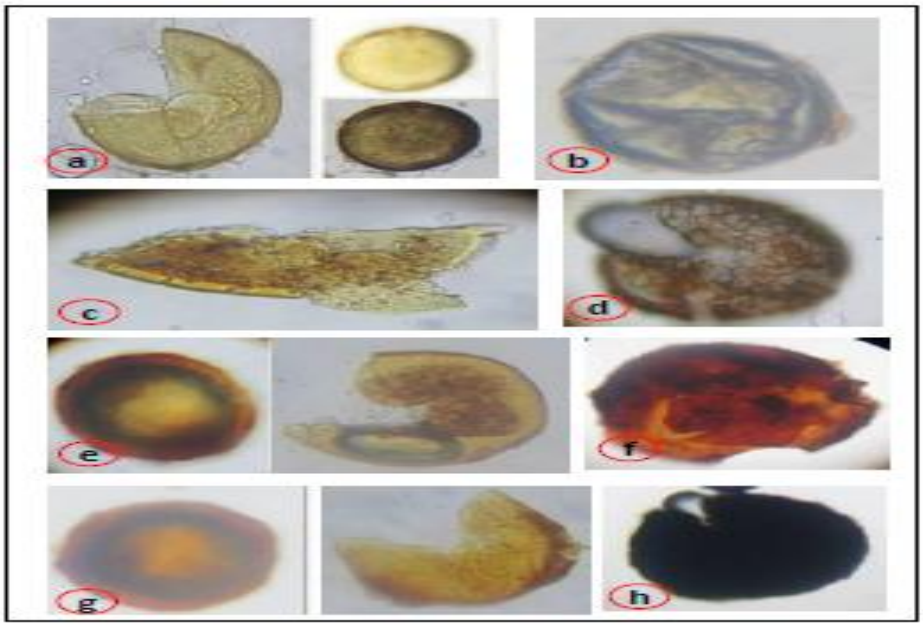

Figure 3. Morphological characteristics AMF spores in rhizosphere soil of endemic and endangered plant *C. media*: a. *Acaulospora scrobiculata* Trappe**; b.** *Acaulospora sporocarpa* Berch.**; c.** *Ambispora callosa* (Sieverd.) Walker, Vestberg & Schüssler**; d.** *Ambispora reticulata* Oehl & Sieverd**; e.** *Gigaspora decipiens* (Hall & Abbot), Bentivenga & Morton**; f.** *Glomus caledonium* (Nicol. & Gerd.) Trappe & Gerd.**; g** *Glomus etunicatum* Becker & Gerd.**; h.** *Glomus microaggregatum* Koske, Gemma & Olexia

IV. Conclusion

Mycorrhizal association represents a major factor that needs to be considered in the effort to sustain survival rate of endangered plant species. However, an elementary prerequisite for any endorsement of such symbiosis is a basic understanding of the occurrence and diversity of AMF in affected ecosystems or with plant taxa, because diversity of AMF has significant ecological role. Under such circumstances, our findings are providing the primary understandings on mycorrhizal status of endemic and endangered plant *C. media.* This information can be effectively utilized to produce native AMF inoculum for conservation or re-vegetation programs in order to maximize the potential benefits that these isolates can provide for the establishment of *C.*

media at threatened habitat in near future. However, to develop and introduce successful method for the application of AMF in *C. media* conservation research must be extended to investigate plant - fungus possible dependency in near future.

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