Morphology, anatomy and mycorrhizae in subterranean parts of *Zeuxine gracilis* (Orchidaceae)

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Resumen

Morfología, anatomía y micorrizas en las partes subterráneas de Zeuxine gracilis *(Orchidaceae)*

Zeuxine gracilis (Berda) Bl. es una orquídea terrestre endémica cuya morfología, anatomía y micorrización es desconocida. A partir de plantas colectadas en la región de los Ghats occidentales se investigó: (a) anatomía de la raíz y el rizoma; (b) características de los pelos radiculares y patrones de colonización de micorrizas. Los caracteres más relevantes en raices fueron: ausencia de velamen y espirantosomas; exodermis simple y nueve protoxilemas arqueados. Rizoma con epidermis uniseriada, abundantes espirantosomas en células corticales internas, endodermis con bandas de Caspary y paquetes vasculares biseriados. Se descubrió la presencia de hongos micorrícicos tanto en las raíces como en los rizomas. Su entrada es principalmente a través de pelos radiculares y epidermis del rizoma. Los hongos forman pelotones y células monilioides en el cótex radicular. Ocasionalmente aparecieron micorrízas arbusculares (AM), caracterizadas por hifas sifonales, vesículas y esporas. La falta de arbúsculos en *Z.gracilis* indica que AM no son funcionales.

Palabras clave: Hongos micorrícicos arbusculares, Células monilioides, Pelotones, Rizoma, Raíz.

Abstract

Zeuxine gracilis (Berda) Bl., is an endemic, terrestrial green orchid whose morphology, anatomy and mycorrhizal status is unknown. So we investigated: (a) root and rhizome anatomy; (b) root hair characteristics and mycorrhizal colonization patterns in *Z. gracilis* plants collected from Western Ghats region of southern India. The prominent anatomical characters in the roots were: absence of velamen, spiranthosomes, and the presence of single layered exodermis and nine arched protoxylem. The rhizome had an uniseriate epidermis, abundant spiranthosomes in the inner cortical cells, a distinct endodermis with casparian strips and biseriate vascular bundles. The presence of fungi both in the roots and rhizomes was revealed. The entry of fungi was chiefly through root hairs and through epidermis in the rhizome. Fungi formed pelotons and monilioid cells in the root cortex. Additionally, arbuscular mycorrhizal (AM) fungi characterized by the presence of aseptate hyphae, vesicles and spores were present occasionally in roots. The lack of arbuscules in *Z. gracilis* indicated the AM to be non functional.

Key words: Arbuscular mycorrhizal fungi, Monilioid cells, Pelotons, Rhizome, Root.

Introduction

Orchidaceae with around 25,000 to 30,000 species is one of the largest families among flowering plants. One of the peculiar features of this family includes the production of dust-like non endospermous seeds. Mode of seed germination and plant development in Orchidaceae is unique among flowering plants, which require association with the mycorrhizal fungi. Orchids have varied life-forms like epiphytes, lithophytes, or terrestrial. Among these varied life-forms terrestrial orchids constitute less than one fourth number of the total orchid species.

Zeuxine belonging to the subfamily Orchidoideae and tribe Cranichideae is one of the largest genus among Orchidaceae. Taxa in *Zeuxine* are widely distributed in Asia with a few in Africa and Australia. In India *Zeuxine* is represented by 11 species of which *Zeuxine gracilis* (Berda) Bl., and *Zeuxine longilabris* (Lindl.) Benth. ex Hook.f., are found in South India (Hooker 1895). *Zeuxine gracilis* is a perennial succulent herb found in shaded woodlands, with brown creeping rhizome, ascending to erect aerial shoot with petiolate velvety green leaves (Abraham & Vatsala 1981). *Zeuxine gracilis* is endemic to South India.

Published work on the anatomy of *Zeuxine* is limited. Stern et al. (1993b) studied the anatomy of *Zeuxine oblonga* R. Rogers & C. White and *Zeuxine strateumatica* (L.) Schltr., while examining the vegetative anatomy and systematics of the subfamily Spiranthoideae in which the tribe Cranichideae was included at that time. These authors found the vascular bundles in *Z. oblonga* and *Z. strateumatica* arranged in one and two series respectively and the absence of stored starch in the former. In this study, Stern et al. (1993b) also reported 'spiranthosomes', a specialized type of amyloplast in many species of Cranichideae including *Z. strateumatica*.

Under natural conditions orchids are associated with mycorrhizal fungi belonging to basidiomycota and ascomycota (Elena et al. 2010). The fungal hyphae invade the cortical cells and form tightly interwoven coils called pelotons characteristic of orchid mycorrhizae (Smith & Read 2008). Reports in literature also indicate the association of arbuscular mycorrhizal (AM) fungi (Glomeromycota) with terrestrial orchids. Hall (1976) reported AM-like colonization in the terrestrial orchid *Corybas macranthus* (Hook.f.) Reichb.f., from New Zealand. Raman & Nagarajan (1999) surveyed the occurrence of mycorrhizae in six epiphytic and five terrestrial orchids of Kodaikanal tropical forest of Western Ghats, South India. They found exclusive occurrence of AM associations in all five terrestrial orchids examined. Information on mycorrhizal association in *Zeuxine* is limited. Burgeff (1932) reported mycorrhizal association in *Zeuxine clandestine* Bl., *Zeuxine* sp., and *Zeuxine purpurascens* Bl., and indicated a trend from the autotrophic condition to saprophytism among these species which were strongly related to mycorrhizal dependence. Porter (1942) reported mycorrhizal association in *Z. strateumatica* growing in Florida, USA. Apart from these there appear to be no information on mycorrhizal status of other species of *Zeuxine*.

The objective of the present study was to investigate the morphology and anatomy of roots and rhizomes of *Z. gracilis* and also to record the mycorrhizal incidence and morphology.

Material and methods

Fresh plant materials of *Z. gracilis* for our study were obtained from Top Slip, Indira Gandhi Wildlife Sanctuary and National Park, Tamil Nadu, India. This site lies in the Western Ghats south of the Palaghat Gap, known as Anaimalais with an area of 958 sq. km. The area lies between 10.13'-10.33'N and 76.49'-77.21'E, 800 m a.s.l. Rainfall varies between 800-4500 mm.

Fresh plant materials of five individuals collected during May 2011, were preserved in FAA (9 parts 70% ethanol, 0.5 parts formalin and 0.5 parts glacial acetic acid) for 24h and stored in 70% ethanol before sectioning. Transverse sections of roots and rhizomes were prepared by free hand sections and stained with safranin or trypan blue. Observations were made using an Olympus BX-51 microscope and images were recorded using Proges 2 camera.

For mycorrhizal assessment the roots and rhizomes were cut into 1-cm long bits, cleared in 2.5% aqueous potassium hydroxide at 90 ºC for 45 min., washed in running water, acidified with 5 N HCl and stained with 0.05% trypan blue in lactoglycerol. The extent of mycorrhizal coloniza-

tion was estimated according to magnified intersection method (McGonigle et al. 1990). Pelotons were considered intact if the constituting hyphae were distinguishable and considered degenerating if the fungal hyphae could not be clearly distinguished or found as an amorphous mass. Twenty 1-cm long root bits were floated in water on a glass slide to measure root thickness and to count root hairs. Length and width of 50 root hairs, and fungal variables (pelotons, monilioid cells, and intracellular hyphae) were measured using an ocular micrometer. We measured only 10 to 12 AM fungal vesicles and spores due to their infrequent occurrence. Measurements were presented as (minimum value-) mean ±S.E. (-maximum value).

Results

Root

Roots arise from the nodal region, off-white, (1.98-) 2.12 ± 0.001 (-2.29) mm in diameter (Fig. 1A). At the point of attachment to the rhizome, the roots were enlarged (2.81-) 3.02 ± 0.16 (- 3.33) mm. Epidermis uniseriate bearing unicellular hairs numbering (7-) 20.06 ± 2.77 (-36) mm^{-1} of root (Fig. 1B). Root hair's single, unbranched cells (86-) 191.80 ± 19.73 (-270) µm long and 3-4 µm wide. Root hairs were swollen at the base (Fig. 1C). Exodermis uniseriate with spirally thickened radial walls (Fig. 1D). Cortex: 16 cells wide, parenchymatic, and distinguishable into two layers. Three to four cells wide cortical layer subtending the exodermis constitute the outer layer of the cortex. This layer is composed of smaller cells and lacks intercellular spaces, whereas the inner layer is characterized by triangular intercellular spaces. Endodermis uniseriate with thin walls; casparian strips present (Fig. 1G). Pericycle: uniseriate with evenly thin walled cells. Vascular cylinder solitary, nine arched. Conductive strands few celled; vascular elements embedded in parenchymatous cells. Pith cells; polygonal, parenchymatous, lacking intercellular spaces.

Rhizome

Subterranean, smooth, brown (4.17-) 4.42 (- 4.80) mm in diameter. Epidermis uniseriate, cells oval with outer walls thickened. Hypodermis one to two cells wide. Cortex: 18-20 cells wide, parenchymatous, cells rounded with small triangular intercellular spaces. Cells of the inner cortical layer contain abundant spiranthosomes (Fig. 1E). Endodermis uniseriate, cells tangentially flattened with casparian strips (Fig. 1F). Vascular cylinder 13 arched with xylem elements arranged in two concentric rings. Vascular tissue embedded in the parenchyma. Pith cells: parenchymatous, thin walled, polygonal, with small intercellular spaces.

Mycorrhizal association

Mycorrhizal colonization occurred uniformly throughout the cortex in roots and rhizomes (Fig. 2A). Fungal hyphae entered the roots by penetrating root hairs or the rhizodermal cells in roots and rhizomes (Fig. 2B). Although single fungal hypha entered each root hair, occasionally more than one could be seen in a root hair (Fig. 2B). Fungal entry into roots or rhizomes was not characterized by the presence of vesicular or appresorial structure. No fungal structures were found in the endodermis or in the stellar portion of roots and rhizomes. Intact pelotons were abundant in the young regions of the roots and rhizomes (Fig. 2C). Colonization in older parts of the roots and rhizomes were disintegrating, the pelotons collapsing into clumps and no recolonization of the host cell was found (Fig. 2F). Sizes of the intact pelotons were (109-) 158.04 ± 10.33 (-264) × (23-) 56.42 ± (-91) µm. The fungal hyphae within the root cortex were, hyaline, smooth, (2-) 3.0 ± 0.58 (-4) µm wide and those of the rhizomes were (6-) 4.67 ± 0.67 (-8) µm wide. Major part of the pelotons in the roots and rhizomes were transformed into monilioid cells (Fig. 2D, E). The dimensions of the fungal monilioid cells were (12-) 14 ± 1.15 (-16) × (8-) 9.33 ± 1.33 (-12) µm in roots, and (14-) 15.33 ± 0.67 (-16) × (10-) 12.67 ± 1.76 (-16) µm in rhizomes. The nucleus of colonized cells was enlarged and distorted (data not shown). Roots of *Z. gracilis* showed a uniform and high degree of colonization of (58-) 67.33 ± 7.42 (-82) % compared to (24-) 28.67 ± 2.90 (- 34) % in the rhizome. In root non-septate intercellular hyphae and intracellular *Glomus*-like vesicles were evident (Fig. 2G). The vesicles were oval (80-) 92.53 ± 6.52 (-105) × (30-) 38.26 ±5.67 (-45) µm. AM fungal spores measuring (45-) 62.01 ± 4.08 (-89) × (50-) 61.92 ± 4.17 (-94) µm were also observed in roots containing abundant

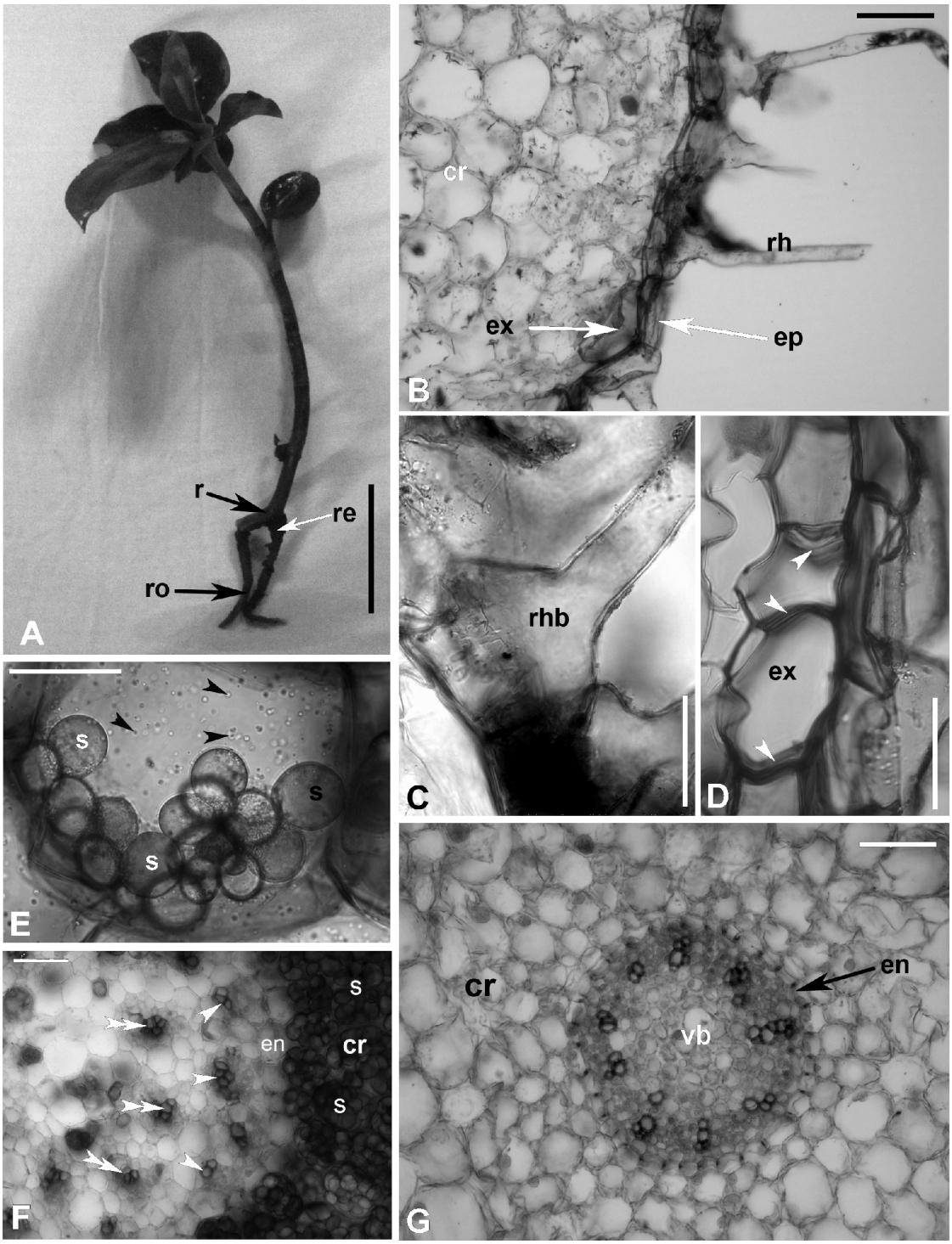

Figura 1. Hábito, raíz y rizoma de *Zeuxine gracilis*. A: Hábito mostrando el rizoma (r), raíz (ro) y alargamiento radicular (re). **B:** Sección transversal de la raíz mostrando los pelos radiculares (rh), la rizodermis (ep), la exodermis (ex) y el córtex (cr). **C:** Un pelo radicular con base hinchada (rhb). **D:** Celulas de la exodermis (ex) en la raíz con espesamientos espirales (flechas). **E:** Espirantosomas (s) en las células corticales del rizoma; las flechas indican granos de almidón procedentes de espirosomas rotos. **F:** Corte transversal del rizoma mostrando el córtex interno con las ceĺulas llenas (cr) de los espirantosomas (s), la endodermis (en) con haces vasculares agrupados en dos series (flechas simples y dobles). **G:** Corte trnasversal de la raíz, cerca del cilindro central, mostrando el córtex (cr), la endodermis con bandas de Caspary (en) y el cilindro vascular (vb). Líneas de escala: A=55cm, B-E = 50µm, F, G = 100 µm.

Figure 1. Habit, root and rhizome of *Zeuxine gracilis*. **A:** Habit showing rhizome (r), root (ro) and root enlargement (re). **B:** Cross-section of the root showing root hairs (rh), rhizodermis (ep), exodermis (ex) and cortex (cr). **C:** A root hair with a swollen base (rhb). **D:** Exodermis cells (ex) in the root with spiral thickenings (arrow heads). **E:** Spiranthosomes (s) in rhizome cortical cell, arrow heads indicate starch grains from ruptured spiranthosomes. **F:** Cross-section of the rhizome showing inner cortex with spiranthosomes (s) filled cells (cr), endodermis (en) with vascular bundles arranged in two series (single and double arrowheads). **G:** Cross-section of the root near the central cylinder showing cortex (cr), endodermis with casparian strips (en) and vascular cylinder (vb). Scale Bars: A=5cm, B-E = 50µm, F, G = 100 µm

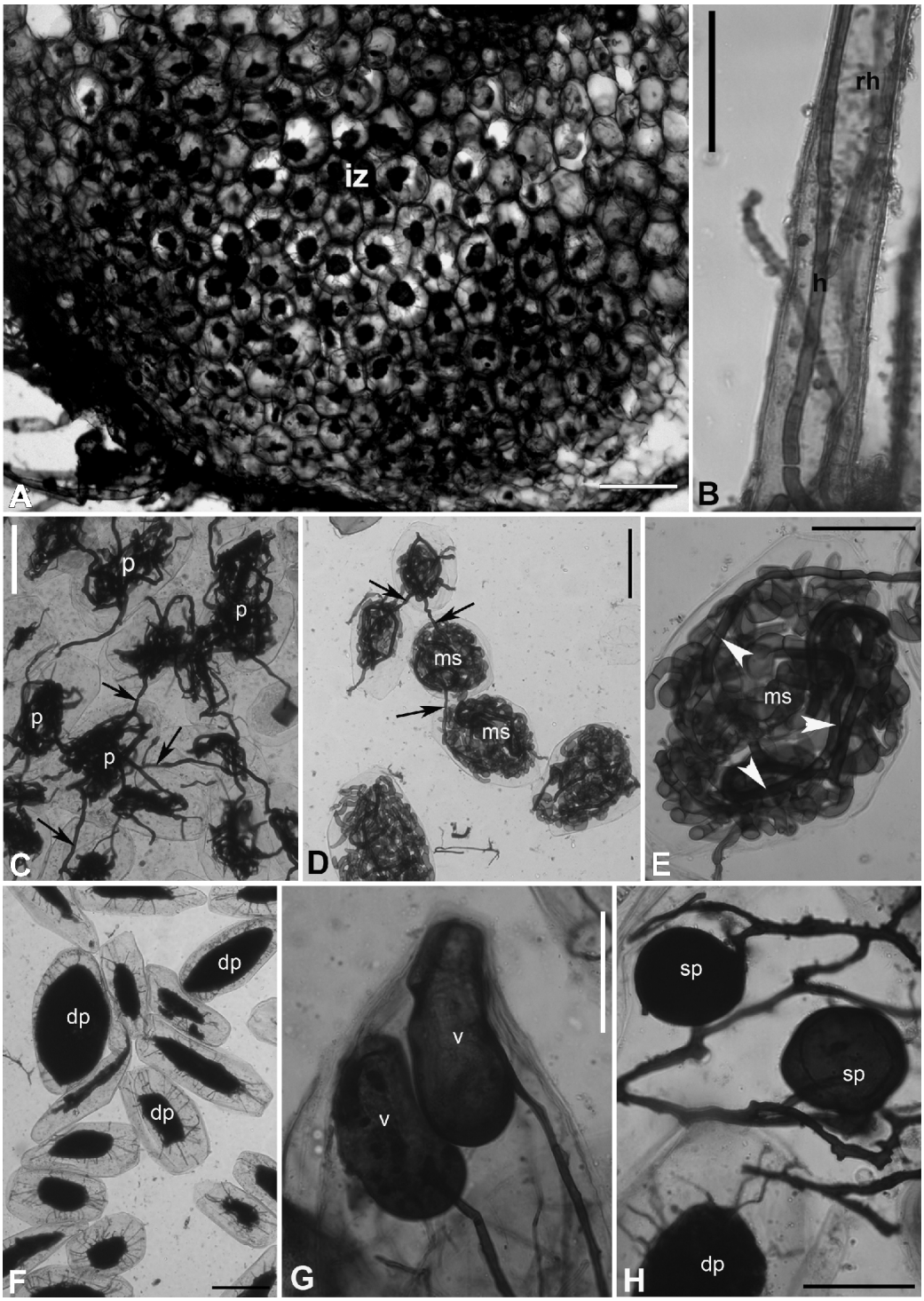

Figura 2. Colonización micorrícica en *Zeuxine gracilis*. **A:** Sección trnasversal del rizoma mostrando la zona de colonización. **B:** Un pelo radicular (rh) con hifa (h). **C:** Pelotones (p) intactos en las células corticales de la raíz. **D:** Células monilioides (ms) e hifas pelotónicas normales (flechas) en rizoma. **F:** Petotones degenerados (dp) en las células corticales de la raíz. **G:** Vesículas (v) de micorrízas arbusculares en las células corticales de la raíz. **H:** Esporas (sp) de micorrízas arbusculares y pelotones degenerados (dp) en raíz. Líneas de escala: A, C, D, F = 100 µm, B, E, G, H = 50 µm

Figure 2. Mycorrhizal colonization in *Zeuxine gracilis*. **A:** Cross-section of the rhizome showing colonization zone (iz). **B:** A root hair (rh) with fungal hyphae (h). **C:** Intact pelotons (p) in root cortical cells. **D:** Monilioid cells (ms) in the cortical cells of rhizome. **E:** Monilioid cells (ms) and normal pelotonic hyphae (arrow heads) in rhizome. **F:** Degenerating pelotons (dp) in root cortical cells. **G:** Vesicles (v) of arbuscular mycorrhizal fungi in root cortical cell. **H:** Spores (sp) of arbuscular mycorrhizal fungi and degenerating peloton (dp) in root. Scale Bars: A, C, D, F = 100 µm, B, E, G, H = 50 µm

degenerating pelotons (Fig. 2H). The extent of such AM fungal colonization was <5% of the root length. Neither vesicles nor spores of AM fungi were observed in rhizomes.

Discussion

Substantial gap in our knowledge on orchid morphology, anatomy and mycorrhizal association persist in spite of several decades of intense research. For example, very limited taxa from each genus have been examined for their anatomy or mycorrhizal status. Root hair characteristics are unknown in many orchids. Root hairs of *Z. gracilis* with wider base resemble the root hairs of *Stenorrhynchos speciosus* (Jacq.) L. C. Rich. ex Spreng., of the same tribe (Stern et al. 1993b). While the roots of *Z. gracilis* resemble *Phaius tankervilliae* (Banks ex L' Herit.) Blume., in their size, the root hairs of *P. tankervilliae* are much longer and thicker compared to *Z. gracilis* (Muthukumar & Sathiyadash 2009). Root hair length and density are controlled by both environmental and genetical factors of the plant (Datta et al. 2011). Among the different environmental factors, nutrient availability appears to be a major factor in determining root hair abundance (Datta et al. 2011). It is well acknowledged that plant with long and dense root hairs absorb more nutrients from the soil than those with relatively short root hairs.

Root anatomy of *Z. gracilis* resembles those of *Z. strateumatica* in the lack of velamen and the presence of nine arched protoxylem arms (Porter 1942, Stern et al. 1993b). There appears to be substantial variation in these characters in the genus *Zeuxine*. For example, one layered velamen and 14 armed protoxylem have been reported in *Z. oblonga* (Stern et al. 1993b). Our observation of an uniseriate exodermis with scalariform thickenings on the radial walls of *Z. gracilis* agrees with the observations of Figueroa et al. (2008) in several members of Cranichideae. The cortical layers in the root are within the range of 8-25 reported for Cranichideae. However, the heterogeneous cortex resembles those of *Goodyera pubescens* (Willd.) R. Br. (Stern et al. 1993b). Stern et al. (1993b) indicated the embedment of one or two strands of vascular tissue in the pith region of *Z. strateumatica*, whereas such strands were not evident in *Z. gracilis*. The supraendodermal spaces that has been reported in roots of several members of Cranichideae (Figueroa et al. 2008) were absent in roots of *Z. gracilis*.

The arrangement of vascular bundles in two series in the rhizome of *Z. gracilis* is in line with a previous study by Stern et al. (1993b), who also reported a similar arrangement in the aerial stems of *Z. oblonga*. The rhizome of *Z. gracilis* differs from the aerial stems of Cranichideae members in the presence of a distinct endodermis with casparian strips. This is in contrast to the aerial stems of *Zeuxine* spp., where an identifiable endodermal layer or endodermis is absent (Stern et al. 1993b). The rhizome of *Erythrodes hirtella* (Sw.) Fawc. & Rendle also has a true endodermis with casparian strips though, the aerial stem has only endodermoid layer (Stern et al. 1993b). Although raphides have been reported in cortical cells of *Z. oblonga*, these were absent in the cortex of *Z. gracilis*.

Stern et al. (1993a) described spiranthosomes, a unique form of amyloplasts from cortical cells of Spiranthoideae. Spiranthosomes were described in the root cortical cells of several tribes of the sub family Spiranthoideae, including Cranichideae. In *Z. gracilis*, we did not observe any spiranthosomes in the cortical cells of the root. There appears to be some inconsistency in the occurrence of spiranthosomes in the roots of *Zeuxine* spp. For example, Stern et al. (1993a,b) noted the presence of spiranthosomes in roots of *Z. strateumatica*, but not in *Z. oblonga*. However, spiranthosomes occurs in abundance in the inner cortical cells of the rhizomes in *Z. gracilis*. This in accordance with Schmucker (1927) who also reported the occurrence of spherical bodies resembling spiranthosomes in the rhizomes of *Haemaria discolor* A. Rich. Spiranthosomes have also been reported in the aerial stems of *Vrydagzynea pachyceras* Schltr., *Prescottia stachyodes* (Sw.) Lindl., and *Pelexia laxa* (Poepp. & Endl.) Lindl. (Stern et al. 1993b).

The entry of fungi into roots and rhizomes is similar to those observed in earlier studies (Senthilkumar et al. 2001, Látr et al. 2008, Muthukumar & Sathiyadash 2009). The presence of mycorrhizal fungi in the rhizome of *Z. gracilis* agrees with the studies (Warcup 1985, Yagame et al. 2008) where the occasional presence of mycorrhizal fungi had been found in this plant part.

Porter (1942) also found abundant colonization in the rhizomes of *in vitro* raised *Z. strateumatica* plantlets. In contrast, Látr et al. (2008) reported the lack of mycorrhizal fungi in unbranched rhizomes of *Cephalanthera longifolia* (L.) Fritsch. The fungi formed monilioid cells in the cortical cells of rhizomes and roots in addition to pelotons. An important event in the orchid mycorrhizal association is the lysis of the fungal pelotons. Lysis is initiated sequentially in the oldest colonized cells followed by cells that have been subsequently colonized (Senthilkumar et al. 2001). Peloton lysis in *Z. gracilis* occurs in distinct zones and not randomly as reported in *Spathoglottis plicata* Blume (Senthilkumar et al. 2001). Like many autotrophic orchids, the mycorrhiza in *Z. gracilis* is of tolypophagy in which the fungal hyphae are digested, and the organic products from the fungal mycelium are transferred to the plant (Burgeff 1932). Recolonization of cells after peloton digestion has been observed in earlier studies. Senthilkumar et al. (2001) reported the presence of pelotons of up to three generations in the root cortical cells of *S. plicata*. Kristiansen et al. (2001) also reported recolonization of *Neuwiedia veratrifolia* protocorm cells containing disintegrating pelotons. However, such recolonization of cells containing disintegrating pelotons was not observed in the present study. Average colonization in *Z. gracilis* is higher compared to those reported for other ground orchids like *P. tankervilliae* (51%), *S. plicata* (57%), *C. longifolia* (<5%) and *Dactylorhiza majalis* (Rchb.f.) P.F. Hunt & Summerh. (12%) (Senthilkumar et al. 2001, Látr et al. 2008, Muthukumar & Sathiyadash 2009). Katiyar et al. (1985) recorded a colonization density of 69-97% in the 12 species of terrestrial orchids they examined from north eastern India. As methods used for estimating colonization in these studies are different comparisons are difficult.

Mycorrhizal fungi that colonize the roots of orchids belong to basidiomycota or ascomycota (Elena et al. 2010). However, the presence of AM fungal structures in roots of *Z. gracilis* is in accordance with studies (Hall 1976, Raman & Nagarajan 1999) where their presence in orchid roots was noted. The lack of arbuscules in AM roots of *Z. gracilis* is in accordance with Hall (1976) who also found only *Glomus*-type vesicles and hyphae, but not arbuscules in the terrestrial orchid *C. macranthus*. However, these observations contradict those of Raman & Nagarajan (1999) who reported the occurrence of arbuscules in *Anoectochilus elatus* Lindl., and *Habenaria elliptica* Wight growing in the Kodaikanal forests of the Western Ghats in southern India. Though the absence of arbuscules suggests that AM in *Z. gracilis* is non-functional, further observations involving more number of plants and over different seasons are essential to ascertain this. DeMars & Boerner (1995) proposed that the presence of AM in non-host root is the primary consequence of progressive root senescence and colonization spread from adjacent AM plants. In addition, senescing roots and organic matter are known to act as a niche for AM fungal sporulation (Nasim 2010).

In conclusion, our observations on the root and rhizome anatomy of *Z. gracilis* agree in general with observations on anatomy in members of Cranichideae by Stern et al. (1993b) and Figueroa et al. (2008). Nevertheless, the role of root hairs in plant nutrient uptake in terrestrial orchids needs to be explored. Similarly, the role of mycorrhizal fungi on plant growth and nutrient uptake in *Z. gracilis* needs to be ascertained experimentally.

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