

Morphology, anatomy, and mycorrhizal fungi colonization in roots of epiphytic orchids of Sempu Island, East Java, Indonesia

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Abstract. Nurfadilah S, Yulia ND, Ariyanti EE. 2016. Morphology, anatomy, and mycorrhizal fungi colonisation in roots of epiphytic orchids of Sempu Island, East Java, Indonesia. *Biodiversitas* 17: 592-603. Roots of orchids have important role for survival, adaptation, water and nutrient absorption, and as a place of symbiosis with mycorrhizal fungi. The present study aimed to investigate the morphology, anatomy, and mycorrhizal status in roots of orchids of Sempu Island, Indonesia (*Ascochilus emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*), in relation to their adaptation to their habitat of coastal forests of Sempu Island. These orchids have different morphological characters; *Ascochilus emarginatus* and *Thrixspermum subulatum* are leafy orchids, while *Taeniophyllum biocellatum* is a leafless orchid. The results showed that all orchids have small number of velamen layers (1-2 layers) as an adaptation to the relatively humid condition. Cell wall thickenings of velamen, exodermis, and endodermis are structural adaptation of all orchids to the relatively high intensity of illumination, to reduce water loss because of transpiration. Mycorrhizal fungi colonization which is important for nutrient acquisition occurs in cortical cells. All orchids have differences in their cell shape, size, and specific characters, such as chloroplasts. The leafless *Taeniophyllum biocellatum* has many chloroplasts in the cortical root cells that support the photosynthesis process, while *A. emarginatus* and *T. subulatum* are lack of chloroplasts in their cortical root cells.

Keywords: Anatomy, morphology, orchids, Sempu Island, symbiotic association

INTRODUCTION

Orchidaceae is one of the most diverse and the greatest plant families containing 25,000-30,000 species worldwide. They have various morphologies with specialized features that allow the family to thrive in different environments and to occupy diverse habitat types. The morphological structures of vegetative organs are specifically variable among species (Dressler 1993). Some orchids are leafy, while some others are leafless.

Root of orchids is a vital vegetative part that has important role for survival, adaptation, water absorption, nutrient acquisition, and as a place of symbiosis with mycorrhizal fungi. There is specialization in the anatomical structure of orchid roots consisting of components that support the function of the roots and to adapt to specific environments (Figueroa et al. 2008; Moreira et al. 2013). For example, large number of velamen layers is related to the orchids growing in arid and dry areas, while small number of velamen layers is associated with orchids from relatively humid areas (Dycus and Knudson 1957; Sanford and Adanlawo 1973). Another specialization in orchid roots is the colonization of mycorrhizal fungi in the cortical cells of orchid roots. Orchidaceae is characterized by its symbiotic association with mycorrhizal fungi partly or in its entire life cycle (from early development to the adult stage of orchids). Orchids are highly dependent on the mycorrhizal fungi as it is a nutrient supplier for the orchids. Mycorrhizal fungi are known to have capacity to absorb nutrients from soil or other substrates and transfer a

proportion of the nutrients to the orchids (Smith et al. 1994; Rasmussen 2002; Nurfadilah et al. 2013). In the early development of orchids, mycorrhizal fungi colonize orchid seeds that are tiny and lack of nutrient reserves. Colonization of mycorrhizal fungi in the orchid seeds is important for seed germination and seedling development (Arditti 1991; Dearnaley and McGee 1996; Swarts and Dixon 2009; Steinfert et al. 2010). In the adult stage of orchids, mycorrhizal fungi colonize the orchid roots on the organ that contacts with soil or other substrates, in which mycorrhizal fungi live and grow (Brundrett 1991; Batty et al. 2002; Kristiansen et al. 2004; Stark et al. 2009; Steinfert et al. 2010; Sakamoto et al. 2015). The mycorrhizal fungi facilitate to absorb nutrients from soil or substrates for more effective nutrient uptake.

The aim of the present study was to investigate the morphology, anatomy, and mycorrhizal status in roots of epiphytic orchids of Sempu Island (*Ascochilus emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*). These orchids have different morphological characters; *Ascochilus emarginatus* and *Thrixspermum subulatum* are leafy orchids, while *Taeniophyllum biocellatum* is a leafless orchid. Sempu Island is a small island off the south coast of East Java province, Indonesia and has an area of 877 ha. Little is known about the biology and ecology in this small island, especially the biology and ecology of orchids. It is administratively located in Malang Regency, East Java. The coastline is mainly composed of limestone cliffs, off the southern part of East Java in Indian Ocean.

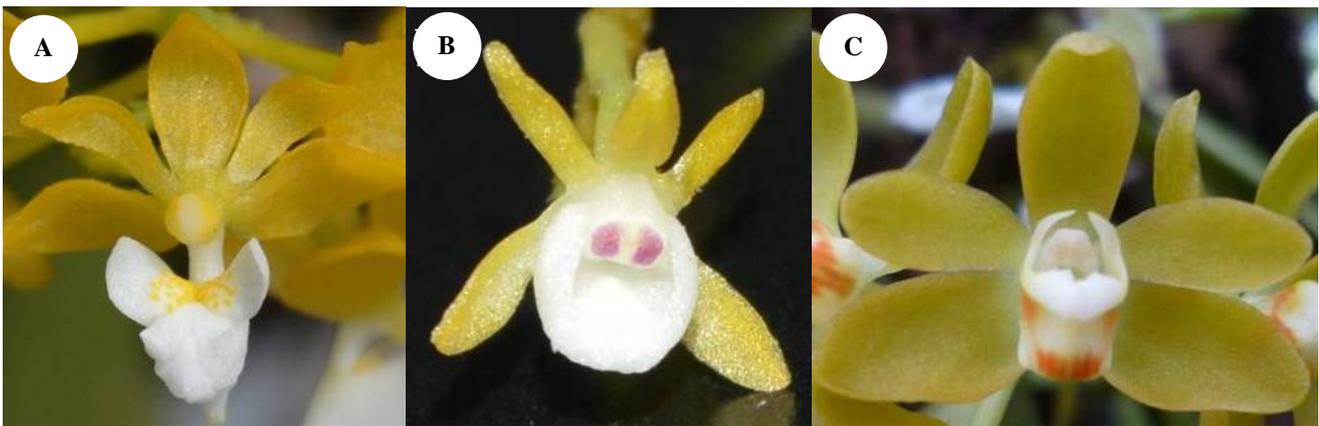


Figure 1. Orchids of Sempu Island, East Java. A. *Ascochilus emarginatus*, B. *Taeniophyllum biocellatum*, C. *Thrixspermum subulatum*

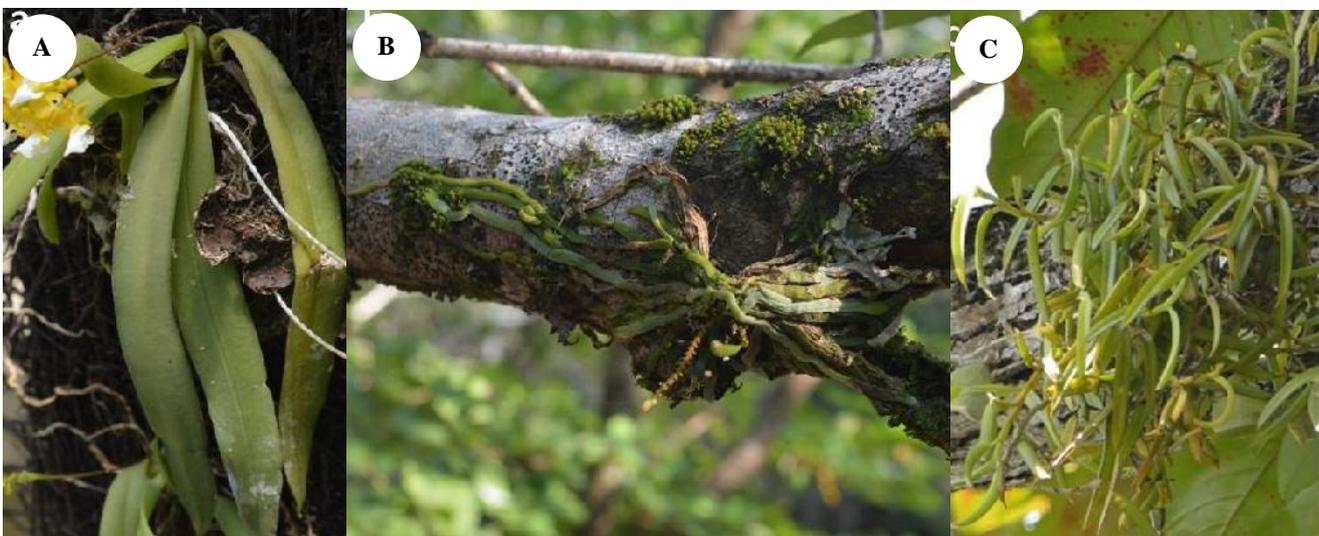


Figure 2. The vegetative organs of orchids of Sempu Island. A. The leafy orchid *Ascochilus emarginatus*, B. The leafless orchid *Taeniophyllum biocellatum*, C. The leafy orchid *Thrixspermum subulatum*

The ecosystems are characterized by coastal forests. The island is a nature reserve under the Ministry of Forestry. Present study also aimed to reveal biology and ecology of orchids in this small island to support orchid conservation programs.

MATERIALS AND METHODS

Materials

Roots of orchids of Sempu Island (*Ascochilus emarginatus* (Blume) Schuit, *Taeniophyllum biocellatum* J. J. Sm., and *Thrixspermum subulatum*), FAA (Formaldehyde Acetic Acid), 70% ethanol, 0.01% Fuchsin acid, glycerol, microtome, object glass, cover glass.

Methods

Roots of epiphytic orchids from areas of Air Tawar and Teluk Semut of Sempu Island (*Ascochilus emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*)

were collected. These areas are relatively humid. The orchids grow on host trees in illuminated areas.

The orchid roots were fixed in FAA (Formaldehyde Acetic Acid) for several days, and transferred to 70% ethanol for several days. The roots were sectioned transversally with a microtome. The slices were stained with 0.01% Fuchsin acid or Methylene blue for one night, mounted in glycerol, and observed under microscope.

The characterization is based on the morphological features, anatomical characters and mycorrhizal fungi colonization. The morphological features of all orchids were characterized. Anatomical characters of velamen, exodermis, passage cells, tilosomes, cortex, endodermis, vascular bundles, and other characters (such as the presence of tilosomes, chloroplasts, and supraendodermal cells) were observed under light microscope. The cell size of orchid roots was measured using micrometer. Mycorrhizal fungi colonization was also screened from the outer part to the inner part of orchids.

RESULTS AND DISCUSSION

The results showed that all orchids of Sempu Island (*Ascochilus emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*) in the study had similarity and difference in the morphological and anatomical characters (Table 1, 2, and 3). The study also demonstrated that mycorrhizal fungi were present in orchid roots, colonized orchid roots and formed symbiotic association with the orchid roots. The anatomical features of roots of these orchids are related to their habitat in the coastal forests of Sempu Island.

Morphological characters

All orchids of Sempu Island in the study has morphological similarity in the presence of roots, while differences of morphological characters among orchids are clearly seen in the presence and absence of leaves, the shape and the color of the roots (Table 1). Leaves are present in *Ascochilus emarginatus* and *Thrixspermum subulatum*, while they are absent in *Taeniophyllum biocellatum*. Although leaves are absent in *T. biocellatum*, it has green roots, indicating the presence of the chloroplasts in its roots. Chloroplast is known as an essential and indispensable component for photosynthesis. The presence of chloroplasts in *T. biocellatum* is confirmed in the anatomical characters of roots of *T. biocellatum* (Table 2; Figure 4.D). *Ascochilus emarginatus* and *T. subulatum* have white greyish colored roots. Their anatomical characters show that chloroplast cells are not clear or absent in their roots.

Anatomical characters of roots of epiphytic orchids

The anatomical organization of roots of orchids of Sempu Island showed that components forming roots consist of velamen, exodermis with the passage cells, cortex, endodermis, vascular bundles, and pith (Table 2). There are specific characters, such as chloroplast, spiral thickening, and supraendodermal cells for particular orchid species (Table 2).

Ascochilus emarginatus

The anatomical characters of *A. emarginatus* root showed that velamen of *A. emarginatus* is uniseriate with cell wall thickening, polygonal shaped cells. There was no epivelamen (outward extension of velamen cells) (Table 2). The exodermis is a single layer with passage cells (smaller cells than exodermal cells, located between exodermal cells). The shape of the exodermis cells is elliptical. The exodermis had wall cell thickening. The cortex had six layers that can be divided into two types of cortex (outer cortex that had smaller cell size (three layers) and inner cortex that had bigger cell size (three layers)). It has rounded to polygonal shaped cortical cells. There were specific characters in *A. emarginatus* root; the presence of spiral thickening in the cortical cells and supraendodermal cells above endodermis (Figure 3.B and 3.E). Mycorrhizal fungi (pelotons; the hyphae of fungi penetrating orchid roots forming a coiled configuration) were present in cortical cells (Figure 3.D). Endodermis is 1 layer with O

Table 1. Comparative morphological characters of three orchids of Sempu Island

Anatomical characters	<i>Ascochilus emarginatus</i>	<i>Taeniophyllum biocellatum</i>	<i>Thrixspermum subulatum</i>
Leaf	Yes	No	Yes
Root	Yes	Yes	Yes
Shape	Cylindrical	Flattened	Cylindrical
Color	White grayish	Green	White grayish

Table 2. Anatomical characters (transverse section) of the roots of orchids of Sempu Island

Anatomical characters	<i>Ascochilus emarginatus</i>	<i>Taeniophyllum biocellatum</i>	<i>Thrixspermum subulatum</i>
Epivelamen			
Epivelamen layer	-	1 layer	1 layer
Epivelamen cell shape	-	Rectangular	Round
Epivelamen cell size			
Velamen			
Velamen layer	1 layer	1 layer	1 layer
Velamen cell shape	polygonal	Rectangular	polygonal elongate; Yes
Velamen thickening	Yes	Yes	Yes
Exodermis			
Exodermis layer	1 layer	1 layer	1 layer
Exodermis cell shape	Ellips	Polygonal	Ellips to polygonal
Exodermis cell size			O
Exodermis thickening			
Passage cell			
Passage cell	Yes	Yes	Yes
Tilosomes			
Tilosomes	Not clear	Not clear	Yes
Type of tilosomes	-	-	-
Cortex			
Outer cortex cell layer	3 layers	2 layers	2 layers
Outer cortex cell shape	Round to polygonal	Polygonal	Polygonal
Outer cortex cell size			
Inner cortex layer	3 layers	5 layers	6 layers
Inner cortex cell shape	Round to polygonal	Round to polygonal	Polygonal
Inner cell cortex size			
Total cortex layer	6 layers	7 layers	8 layers
Width of cortex			
Specific characters			
Chloroplast	No	Yes	No
Spiral thickening	Yes	No	No
Supra endodermal cell	Yes	No	No
Mycorrhizal fungi colonization	Yes	Yes	Yes
Endodermis			
Endodermis	1 layer	1 layer	1 layer
Endodermis thickening	O	O	O
Pericycle	1 layer	1 layer	1 layer
Vascular bundles			
Vascular bundles (archs)	8	6	20
Pith			
Pith	Parenchymatous	Parenchymatous	Parenchymatous

cell wall thickening. It has 1 layer of pericycle. There were 8 archs of vascular bundles composed of phloems and xylems that were embedded in the sclerenchymatous tissues. Pith is parenchymatous (Figure 3).

Taeniophyllum biocellatum

Transverse section of *T. biocellatum* root revealed that velamen is uniseriated with wall thickenings. There was a single layered of epivelamen (the extension of velamen to out side). Exodermis is one-layered with cell wall thickening, and polygonal-shaped cells. The passage cells are smaller than exodermal cells and have tilosomes (thickening above the passage cells). There were two types of cortical cells, outer cortical cells (2 layers), and inner cortical cells (5 layers). The shape of cortex is polygonal, with cell wall thickenings. Chloroplasts were present in cortical cells (Figure 4.D). Endodermis is uniseriate with cell wall thickenings. 1 layered pericycle. Vascular bundles with 6 archs consisted of phloem and xylem that were embedded in the sclerenchymatous tissues. Pith is parenchymatous (Figure 4).

Thrixspermum subulatum

Thrixspermum subulatum had uniseriate velamen periclinally with 1 layer epivelamen. The shape of velamen was polygonal to elongate, with cell wall thickenings. Exodermis was 1 layer with passage cells. The shape of exodermal cells was elliptical to polygonal. The exoermis had O cell wall thickening. Passage cells had tilosomes. Two types of cortex; outer cortex (2 layers) and inner cortex (6 layers). Endodermis was 1 layer with O thickening. Pericycle 1 layer. Vascular bundles 20 archs were composed of phloems and xylems that were embedded in the sclerenchymatous tissues. Pith was parenchymatous.

Comparison between anatomical characters of three species

Orchids of Sempu Island (*Ascochilus emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*) had similarity and difference in their anatomical features (Table 2 and Table 3).

Velamen

Velamen is the outermost of orchid roots. Components of velamen cell consist of cellulose with various proportions of lignin and suberin. The main functions of velamen are mechanical protection, water and nutrient absorption, reduction of transpiration and water loss, and infra red reflection (Dycus and Knudson 1957; Benzing et al. 1982, 1983; Pridgeon 1986; Moreira et al. 2013).

The orchids in the present study have 1-2 velamen layers (2 velamen layers = 1 velamen layer and 1 epivelamen: the outward extension and the development of velamen). The velamen of these orchids had cell wall thickenings. Other orchids; both epiphytic and terrestrial orchids are reported to have velamen, with various number of velamen layers. The number of velamen layers of

epiphytic orchids was various: *Dichaea cogniauxiana* (2 layers), *Epidendrum secundum* (4-5 layers) (Moreira et al. 2013), *Lueddemannia pescatorei* (11-13 layers), *Acineta densa* (12-15 layers), *Coeliopsis hyacinthosma* (4-5 layers), *Coryanthes macrantha* (7-8 layers), *Gongora galeata* (6-7 layers), *Kegeliella atropilosa* (4-5 layers) (Stern and Whitten 1999), *Catasetum fimbriatum* and *Stanhopea lietzei* (15 layers) (Oliveira and Sajo 1999). Terrestrial orchids also have various number of velamen layers: No velamen in *Zeuxine gracilis* (Muthukumar et al. 2011), single layer of velamen, but was partly replaced with an exodermis in *Neottia nidus-avis*, *Limodorum abortivum*, *Serapias orientalis* (Aybeke 2012). *Ophrys iricolor* and *O. morio* have epivelamen without any velamen (Aybeke et al. 2010). Other terrestrial orchids have a uniseriate velamen, such as *Habenaria rhodocheila* (Stern 1997) *Cranichis cochleata*, *Ponthieva ephippium*, *Goodyera brachyceras*, and *Ludisia discolor* (Figueroa et al. 2008). Other terrestrial orchids are reported to have more than one layer of velamen; such as *Bonatea steudneri* (3-4 layers) (Stern 1997), *Calypso bulbosa* (2 layers), *Tipularia discolor* (4 layers) (Stern and Carlsward 2008), *Sauroglossum nitidum* (9- 10 layers)(Moreira and Isaiaas 2008).

The difference in the number of layers of velamen indicates the adaptation of orchids to specific environments. Orchid species from arid and dry habitats were associated with multilayers of velamen, while orchid species from humid habitats were related to lack velamen or only one layer of velamen (Dycus and Knudson 1957; Sanford and Adanlawo 1973). The epiphytic orchids of Sempu Island in this study (*Ascochilus emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*) had similar number of velamen layers (1-2 layers). The low number of velamen layers of these orchids is related to the habitat condition of these orchids which are relatively humid in the coastal forests of Sempu Island.

All orchids of Sempu Island in the present study had velamen cell wall thickening. Velamen cell wall thickening is the result of suberin impregnation with lignified thickenings (Benzing et al. 1983; Noel 1974). Other orchids (both epiphytic and terrestrial orchids) also showed velamen thickening, while some others did not exhibit velamen cell wall thickening. The epiphytic orchids: *Catasetum fimbriatum*, *C. matogrossense*, *C. schmidtianum*, *C. apolloi*, *C. juruense*, *C. longifolium*, *C. osculatum*, and *C. saccatum* had velamen cell wall thickening (da Silva et al. 2015). Thickened velamen was also reported in other epiphytic orchids; such as *Encyclia patens*, *Sophronitis pumila*, *Polystachia estrellensis* (Moreira and Isaiaas 2008), *Epidendrum secundum* (Moreira et al. 2013). In addition, terrestrial orchids were reported to have conspicuous velamen thickening, such as orchids from tribes Spiranthinae and Prescottiinae (except Pseudocranichis) (Figueroa et al. 2008). However, other orchids showed thin-walled velamen, such as *Dichaea cogniauxiana* (Moreira et al. 2013).

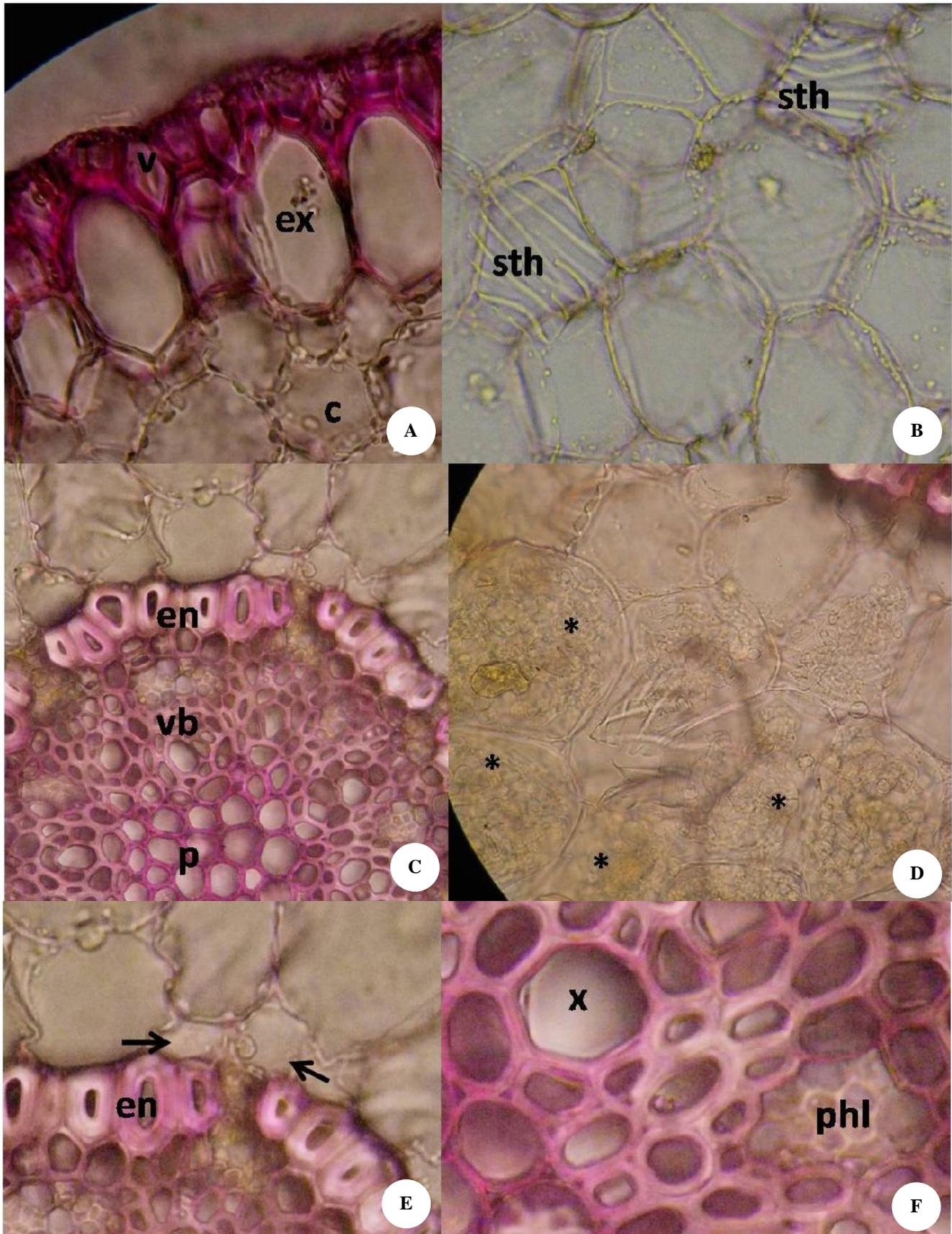


Figure 3. Root anatomy (transverse section) of *Ascochilus emarginatus*. A: epivelamen (ep); exodermis (ex); cortex (c), B: spiral thickening (sth) in cortical cells, C: endodermis (en); vascular bundles (vb); pith (p) D: cortical cells colonized by mycorrhizal fungi are marked with *, E: supraendodermal cells above endodermis (arrow), F: xylem (x); phloem (phl)

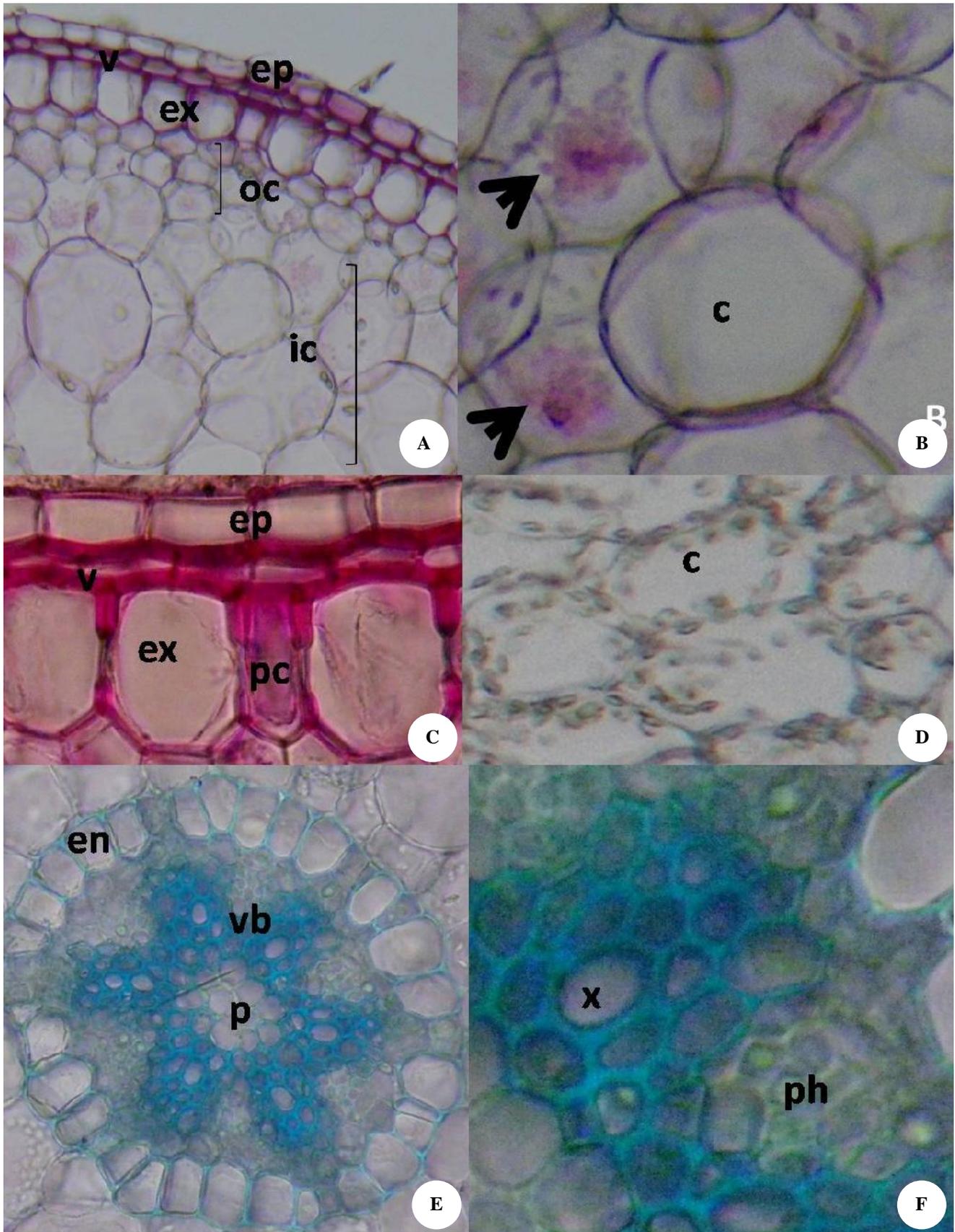


Figure 4. Root anatomy (transverse section) of *Taeniophyllum biocellatum*. A: epivelamen (ep); velamen (v); exodermis (ex); outer cortex (oc); inner cortex (ic); B: cortical cells colonized by mycorrhizal fungi (arrow head) C: passage cell (pc); D: chloroplasts in cortical cells E: endodermis (en), vascular bundles (vb), pith (p); F: xylem (x); phloem (ph)

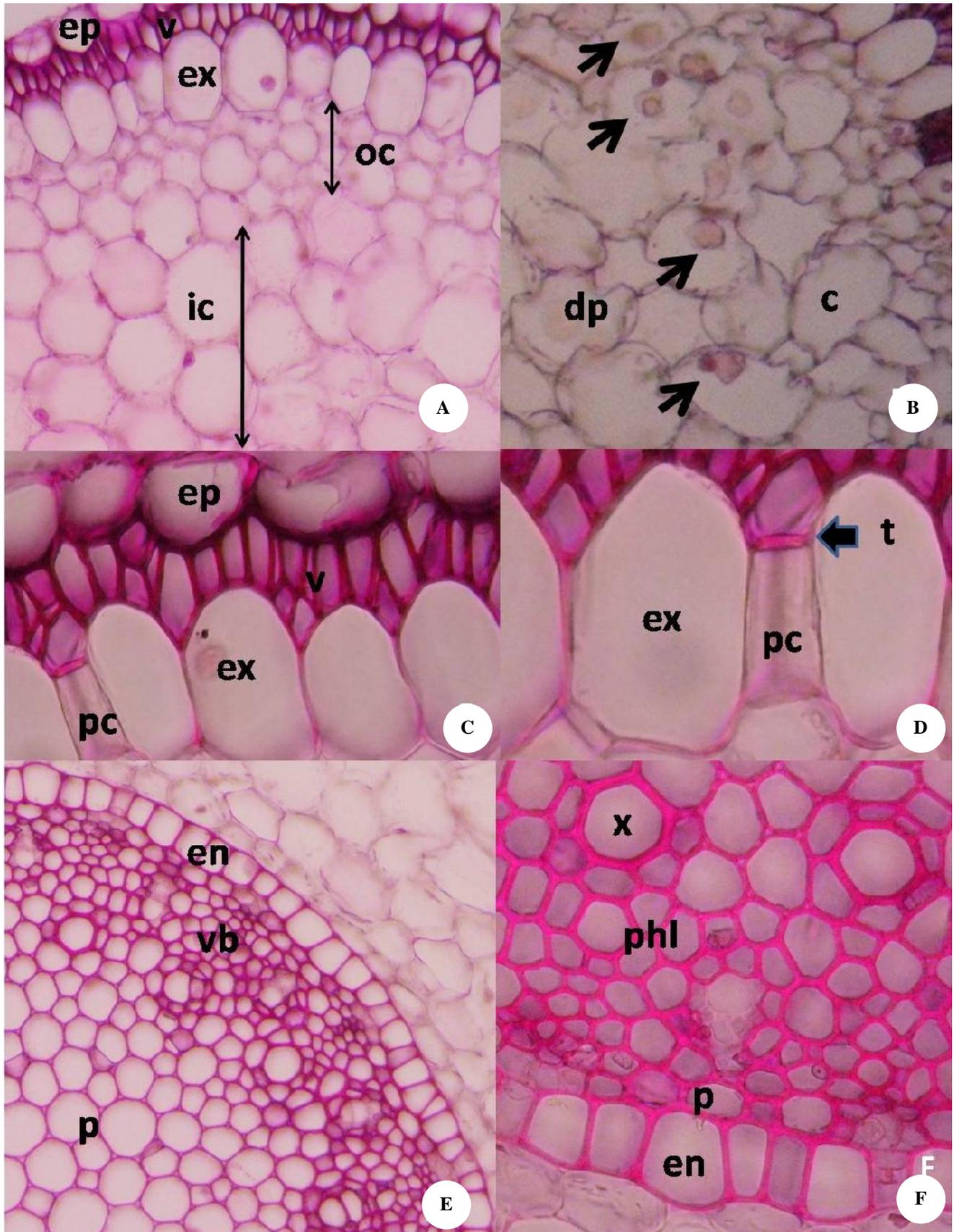


Figure 5. Root anatomy (transverse section) of *Thrixspermum subulatum*. A: epivelamen (ep); velamen (v); exodermis (ex); outer cortex (oc); inner cortex (ic), B: cortical cells colonized by mycorrhizal fungi, degenerative pelotons (dp) (arrow), C: passage cell (pc); exodermis (ex); D: passage cell (pc), tilosome (t) (arrow) E: endodermis (en), vascular bundles (vb), pith (p), F: xylem (x); phloem (phl); pericycle (p), endodermis (en)

The role of velamen cell wall thickening is for mechanical support to avoid water loss (Noel 1974; Benzing et al. 1983). The velamen cell wall thickening in orchids of Sempu Island (*Ascochilus emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*) is related to their habitat and environment. As they grow in illuminated areas and are exposed to high intensity of light, the velamen cell wall thickening is vital to reduce root transpiration and water loss. It is a part of structural adaptation to their habitat in illuminated areas in the coastal forest of Sempu Island.

Exodermis

Below the velamen layers, there is exodermis layer, which is the outer layer of cortex (Engard 1944). The exodermis cell had secondary cell wall thickenings that are empty and dead at maturity (Pridgeon 1986). The exodermis cell wall thickening is caused by lignin and suberin impregnation (Fahn 1990). The function of exodermis cell wall thickening is for mechanical protection against water evaporation, to retain moisture in the cortex, and to control the entrance of mycorrhizae in cortical cells (Benzing et al. 1983; Sanford and Adanlawo 1973; Moreira and Isaiass 2008).

Exodermis of orchids of Sempu Island (*Ascochilus emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*) in the present study is uniseriate. Most orchid species have 1 layer of exodermis. The number of exodermal layers can be more than one layer, such as in some *Ophrys* ranged from 1 to 4 (Aybeke et al. 2010). Orchids of Sempu Island in the present study exhibited exodermis cell wall thickening with various patterns of exodermis cell wall thickening. *Ascochilus emarginatus* and *Taeniophyllum biocellatum* had cell wall thickening, *Thrixspermum subulatum* had O cell wall thickening. Other orchids, both epiphytic and terrestrial orchids also had exodermis thickening with various patterns (Moreira and Isaiass 2008). Other epiphytic orchids were reported to have thickened exodermis; such as *Epidendrum campestre* with thickening, and *Pleurothallis smithiana*, *Vanda discolor*, and *Encyclia calamara* with O thickening (Oliveira and Sajo 1999), *Aerangis confusa*, *A. coriacea*, *A. kirkii*, *Angraecum calceolus*, *A. conchiferum*, *A. teres* with cell wall thickening (Carlsward et al. 2006); *Epidendrum campestre* (Oliveira and Sajo 1999). Furthermore, terrestrial orchids also showed exodermis thickening, such as *Sobralia macrantha* (Benzing et al. 1982); *Cranichis cochleata*, *Ponthieva ephippium*, *Goodyera brachyceras*, and *Ludisia discolor* (Figueroa et al.); *Zeuxine gracilis* (Muthukumar et al. 2011).

Similar to velamen thickening, the cell wall thickening of exodermis of orchids of Sempu Island in the present study is important as the mechanical protection to reduce transpiration and water loss from cortex, as these orchids grow in illuminated areas and exposed to high intensity of light. Such as velamen thickening, exodermis thickening is also a structural adaptation in the coastal forest with high intensity of light (Moreira and Isaiass 2008).

The exodermis size and shape of orchids of Sempu Island in the present study were different (Table 2 and 3). Ellips, polygonal, ellips-polygonal are the shape of exodermis of *Ascochilus emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*, respectively. Other orchids also had various shape of exodermal cells, such as elongate to isodiametric in *Angraecum calceolus*, *Angraecum conchiferum*, *Bolusiella iridifolia*, *Aerangis confusa*, *A. coriacea*, *A. kirkii* (Carlsward et al. 2006).

Passage cells

Between exodermis cells, there are shorter cells that are living and have thin cell wall. Like other orchids, orchids of Sempu Island in the present study also had passage cells that were alternately disposed between exodermis cells. Passage cells in the exodermis layer are important for the passing of water and nutrient, and attracting mycorrhizal fungi (Peterson and Enstone 2006; Senthilkumar et al. 2000).

Tilosomes

Tilosome is the extension from the innermost cell wall of velamen cells attached to the passage cells of exodermis. The function of tilosome is to protect from water loss via root transpiration (Pridgeon et al. 1983). The presence and absence of tilosome is one of key characters in the classification, systematics and phylogenetics of orchids (Figueroa et al. 2008). Of the three species of orchids of Sempu Island in the present study, tilosome was clearly seen in *Thrixspermum subulatum*, while it was not clear or absent in *A. emarginatus* and *Taeniophyllum biocellatum*. The presence or absence of tilosomes was also reported in other orchid species. Tilosomes were present in *Prescottia tubulosa* and *Prescottia stachyodes* (Prescottinae) and in many species of Spiranthinae (Figueroa et al. 2008; some *Ophrys* (Aybeke et al. 2010), while tilosomes were absent in some species of Goodyerinae, Cranichidinae and Manniellinae (Figueroa et al. 2008).

Cortex

Cortex is a tissue beneath exodermis which is formed by thin walled parenchymatous cells with various sizes. Outer cortex layers are composed of small size cells, while inner cortex layers are formed by large size cells (Muthukumar et al. 2011). Number of cortex layers varied among orchid species of Sempu Island in the present study. *Ascochilus emarginatus* had 6 cortex layers (3 outer and 3 inner cortex layers), *Taeniophyllum biocellatum* exhibited 7 cortex layers (2 outer and 5 inner cortex layers), and *Thrixspermum subulatum* possessed 8 cortex layers (2 outer and 6 inner layers). Other orchid species (both epiphytic and terrestrial orchids) were reported to exhibit different number of cortex layers; such as the epiphytic orchids *Dichaea cogniauxiana* (14-16 layers); *Epidendrum secundum* (6-12 layers) (Moreira et al. 2013); while the terrestrial counterparts: *Zeuxine gracilis* (16 layers) (Muthukumar et al. 2011), *Neottia nidus-avis* (9 layers), *Cephalanthera epipactoides* (10-18 layers), *Limodorum abortivum* (18-27 layers), *Platanthera chlorantha* (layers) (Aybeke 2012).

Table 3. Comparison of the anatomical characters of roots of 3 orchids of Sempu Island based on quantitative measurements of cell and layer size of roots

Characters	<i>Ascochilus emarginatus</i>	<i>Taeniophyllum biocellatum</i>	<i>Thrixspermum subulatum</i>
Transverse section width	935,13 ± 37,51 (a)	1787,83 ± 2,94 (c)	1285,5 ± 2,81 (b)
Transverse section length	1021,8 ± 20,13 (b)	605,63 ± 3,67 (a)	1267,53 ± 10,11 (c)
Epivelamen			
Epivelamen length	0 ± 0 (a)	11,58 ± 1,11 (b)	20,85 ± 1,79 (c)
Epivelamen breadth	0 ± 0 (a)	22 ± 1,06 (b)	22 ± 1,32 (b)
Passage cell			
Passage cell length	31,68 ± 1,81 (a)	22,68 ± 1,66 (a)	32,68 ± 1,58 (a)
Passage cell breadth	15,87 ± 1,02 (a)	14,38 ± 1,18 (a)	16,12 ± 1,38 (a)
Velamen			
Velamen length	22,55 ± 1,33 (b)	6,13 ± 0,81 (a)	14,6 ± 1,24 (c)
Velamen breadth	10,93 ± 0,69 (b)	18,57 ± 1,8 (c)	6,4 ± 0,36 (a)
Velamen width	22,77 ± 4,69 (a)	18,57 ± 1,80 (a)	21,03 ± 1,90 (a)
Exodermis length	55,05 ± 2,14 (c)	27,2 ± 1,13 (a)	42,32 ± 1,11 (b)
Exodermis breadth	31,53 ± 1,46 (b)	24,4 ± 3,13 (ab)	22,83 ± 1,05 (a)
Cortex			
Outer cortex length	23,1 ± 1,91 (b)	17,83 ± 0,7 (a)	17,47 ± 0,74 (a)
Outer cortex breadth	21,02 ± 2,17 (a)	20,13 ± 1,47 (a)	18,05 ± 1,41 (a)
Inner cortex length	67,95 ± 3,94 (b)	62,62 ± 5,35 (ab)	45,88 ± 2,59 (a)
Inner cortex breadth	67,93 ± 6,27 (a)	50,6 ± 4,59 (a)	50,92 ± 2,33 (a)
Outer cortex layer	59,68 ± 5,97 (ab)	39,4 ± 4,08 (a)	70,78 ± 6,13 (b)
Inner cortex layer	242,95 ± 8,08 (b)	161,38 ± 8,41 (a)	221,02 ± 12,58 (b)
Stele			
Stele width	201,33 ± 1,61 (b)	108,72 ± 3,44 (a)	511,73 ± 4,97 (c)
Endodermis length	19,22 ± 0,65 (a)	19,58 ± 3,57 (a)	15,68 ± 1,00 (a)
Endodermis breadth	10,8 ± 0,68 (a)	17,23 ± 5,45 (a)	13,7 ± 0,97 (a)
Pith			
Vascular bundle	73,25 ± 4,09 (b)	18,58 ± 1,81 (a)	339,97 ± 3,53 (c)
	8 ± 0 (b)	6 ± 0 (a)	20 ± 0 (c)

Spiral thickening in root cortical cells can be a key character of orchid species. In the present study, *Ascochilus emarginatus* had spiral thickening in the cortex, while *Taeniophyllum biocellatum*, and *Thrixspermum subulatum* did not exhibit the presence of spiral thickening in their cortex. Other orchid species were reported to have spiral thickening in their root cortical cells, such as *Eulophia epidendraea* and *Malaxis acuminata* (Uma et al. 2015), *Catasetum schmidtianum*, and *C. juruense* (da Silva et al. 2015). Leroux et al. (2010) suggested that spiral thickening in cortical cells functions as mechanical protection, prevention from desiccation because of root transpiration, and for more efficient water and nutrient uptake.

In the present study, chloroplasts were present in cortical cells of *Taeniophyllum biocellatum*, but they were absent in cortex of *Ascochilus emarginatus* and *Thrixspermum subulatum*. Chloroplasts contain chlorophyll that is important for photosynthesis. They usually occur in leaf cortical cells. The presence of chloroplasts in root cortical cells of *T. biocellatum* may be related to the life form of *T. biocellatum* that do not have leaves (leafless), and they evolve green roots containing chloroplast for photosynthesis to survive. Chloroplasts were not clear or

absent in root cortex *A. emarginatus* and *Thrixspermum subulatum* may be related to their life form in possessing leaves that contain chloroplasts. The color of roots of *A. emarginatus* and *T. subulatum* were white grayish (Table 1). This indicated there were no chloroplasts, as chloroplasts are associated with green colored parts.

Endodermis

Endodermis is a layer beneath cortical cells that protect the inner parts (vascular bundles and pith). Some orchids have secondary endodermal cell wall thickening, while some others exhibit thin walled endodermis. Epiphytic orchids *Angraecopsis parviflora*, *Microcoelia bulbocalcarata*, *M. corallina* and *M. stolzii* had thin walled endodermis (Carlswald et al. 2006). Terrestrial orchids that had thin walled endodermis included *Zeuxine gracilis* (Muthukumar et al. 2011); *Habenaria arenaria*, *H. cornuta*, *H. odontopetala*, *H. snowdenii*, *Stenoglottis fimbriata*, *S. longifolia*, *S. woodii* (Stern 1997) Other orchids exhibited secondary cell wall thickening of endodermis, such as *Bolusiella batesii*, *B. iridifolia*, *Microcoelia aphylla* (Carlswald et al. 2006). Endodermal thickening also occurred in *Acineta densa*, *Luëddemannia*

pescatorei, *Polycynis gratiosa*, *Stanhopea candida* with endodermal thickening, *Cirrhaea dependens*, *Stanhopea pulla*, *Stanhopea panamensis* (Stern and Whitten 1999). Similar to exodermis, the pattern of cell wall thickening varied, such as β and O thickening.

In the present study, the endodermis of orchids of Sempu Island (*A. emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*) had O cell wall thickening. The function of cell wall thickening in endodermis is similar to that of exodermis and velamen, as mechanical protection and as prevention against water loss because of root transpiration. This feature of endodermal cell wall thickening in orchids of Sempu Island can be an adaptation to their habitat that are exposed to high intensity of light in the coastal forest of Sempu Island.

Supraendodermal spaces were observed above endodermis of *A. emarginatus*, while they were absent in *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*. Supraendodermal spaces are small intercellular spaces that occur outside the endodermis. This feature is also a key character in the classification and phylogenetic information (Figueroa et al. 2008). Some orchid species have supraendodermal spaces, while some others do not. It was reported that some orchids having supraendodermal spaces were *Pseudocranichis thysanochila*, *Aulosepalum pyramidale*, *Mesadenus lucayanus*, *Microthelys constricta*, *Sacoila lanceolata*, while those having no supraendodermal were *Cranichis cochleata*, *Goodyera brachyceras*, *Ludisia discolor*, *Manniella gustavi*, *Prescottia tubulosa* (Figueroa et al. 2008). Most of species having supraendodermal spaces occur in high transpiration areas in seasonally dry habitats (Figueroa et al. 2008). The presence of supraendodermal spaces in roots of *A. emarginatus* indicated for more effective protection against root transpiration as this orchid grow in areas of high intensity of light in the coastal forests of Sempu Island.

Vascular bundles

Vascular bundle is a transport system containing xylem and phloem that are important in the transport of water and nutrients. The number of archs in vascular bundles of orchids of Sempu Island in the present study was different. *Ascochilus emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum* had 8, 6 and 20 archs, respectively. Number of archs in vascular bundles is notably various between orchid species (Oliveira and Sajo 1999). Other orchid species showed different number of archs in the vascular bundles, such as *Neottia nidus-avis* (3 archs), *Cephalanthera epipactoides* (7-11 archs), *Limodorum abortivum*, (9-25) *Platanthera chlorantha* (5-10 archs) (Aybeke 2012).

Pith

Pith of orchid is the central part in roots and is composed of parenchym or sclerenchym. The pith of all orchids of Sempu Island in the present study is parenchymatous. Pith of *Microcoelia corallina* was parenchymatous, while the pith of *Angraecopsis breviloba*, *Microcoelia globulosa*, *Microterangis hildebrandtii* was sclerenchymatous (Carlsward et al. 2006).

Mycorrhizal fungi colonization

The results of the present study showed the presence of mycorrhizal fungi (pelotons) in orchid roots of Sempu Island (*A. emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*) (Figure 3, 4, and 5). There is a process in the colonization of mycorrhizal fungi in orchid roots. The mycorrhizal fungi entered the roots through the velamen layers and moved into the inner layers of the roots (exodermis). The exodermis was not colonized by mycorrhizal fungi. This is related to the thick structure of exodermis because of cell wall thickening (Schreiber and Franke 2011). The mycorrhizal fungi were able to penetrate the exodermis through *passage cells* in the exodermis layer (Senthilkumar et al.2000). Passage cells have thin wall that make them possible to be penetrated by mycorrhizal fungi. Passage cells in the exodermis also have specific function to attract mycorrhizal fungi (Peterson and Enstone 2006). The thickened structure of exodermis is important as mechanical protection and to avoid unwanted compounds such as toxin and pathogen microorganisms. Wanted compounds (ions, nutrients) and symbiotic mycorrhizal fungi were able to penetrate the exodermis through the passage cells in the exodermis layer. After entering the passage cells, mycorrhizal fungi colonized the inner layer (cortex cells) (Oliveira and Sajo 1999; Schreiber and Franke 2001; Moreira and Isaias 2008; Matsuda et al.2009; Muthukumar et al.2011)

The distribution and the pattern of mycorrhizal fungi colonization in roots of *A. emarginatus*, *T. biocellatum*, and *T. subulatum* in the present study were similar. The mycorrhizal fungi only colonized the cortex of the roots. The colonization of mycorrhizal fungi forms a coiled configuration of mycorrhizal fungi hyphae (pelotons) in cortex cells. Pelotons were not found in the endodermis or stele (endodermis, phloem, xylem, and pith). The pelotons were not found in epidermis and exodermis either. Other studies reported that orchids from Japan, India, Brazil, South America, and Europe also showed similar results that colonization of mycorrhizal fungi occurred in cortex cells and was not found in other root layers (Oliveira and Sajo 1999; Senthilkumar et al. 2000; Yagame et al. 2008; Fracchia et al.2009; Látalová and Balaz 2010; Muthukumar et al. 2011; Hadley and Williamson 1972).

In the symbiotic association between orchids and the mycorrhizal fungi, orchids absolutely rely on the mycorrhizal fungi in their entire life cycle, while the mycorrhizal fungi can survive without the orchids as the mycorrhizal fungi are saprophytic that have a capacity to absorb nutrients from soil or other substrates (Smith et al. 1994; Rasmussen 2002; Nurfadilah et al.2013). In the relationship with the orchids, mycorrhizal fungi transfer nutrients to the orchids and obtain photosynthate (carbon) transfer from the orchids (Rasmussen 2002; Cameron et al. 2006).

In the perspective of orchids in the symbiotic association with the mycorrhizal fungi, orchids highly depend on the mycorrhizal fungi from the early growth and development of the orchids to the adult stage. Orchid seeds are tiny and lack of nutrient reserves, thus they need external nutrients for seed germination and seedling

development. The external nutrients are fulfilled by nutrients transferred by the mycorrhizal fungi. While the orchids have a photosynthetic capacity, the symbiotic association still has an important role to maximize the nutrient uptakes (Arditti 1991; Dearnaley 2007; Swarts and Dixon 2009).

Implication for conservation

Understanding the biology and ecology of orchids is important in the conservation of orchids. The present study is one part to increase understanding on the morphology, anatomy of orchids of Sempu Island in relation to the ecology of orchids in the coastal forests of Sempu Island.

The finding of the present study on the symbiotic association between orchids and the mycorrhizal fungi increases understanding of the biology and ecology of orchids. The implication of this finding is important in the management of orchid conservation that the conservation of orchids needs to include the conservation of the mycorrhizal fungi as their associates. Orchids are known highly rely on the mycorrhizal fungi in orchid's entire life cycle. Thus, for the existence and the survival of orchids, the conservation of both orchids and mycorrhizal fungi associates is required.

The present study has demonstrated that the colonization of mycorrhizal fungi of all epiphytic orchids of Sempu Island (*Ascochilus emarginatus*, *Taeniophyllum biocellatum*, and *Thrixspermum subulatum*) was similar. These results indicate the symbiotic association of orchid roots and mycorrhizal fungi. The present study increases understanding of the biology of orchids in Indonesia, in terms of its symbiotic association with mycorrhizal fungi. The implication of this finding is important in the management of orchid conservation that the conservation of orchids needs to include the conservation of the mycorrhizal fungi associates.

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