Anatomy of the invasive orchid *Oeceoclades maculata*: ecological implications

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Oeceoclades maculata is the most successful invasive orchid in the Neotropics. The anatomy of the vegetative organs, peduncle and seeds of *O. maculata* was characterized to identify features of possible physiological and ecological importance. Plants from four locations in Soconusco, Chiapas, Mexico were selected. Transverse, longitudinal and paradermal sections of vegetative organs were observed using light and scanning electron microscopes. *Oeceoclades maculata* has amphistomatous leaves, with smooth and a thin to slightly thickened cuticle, a single-layered epidermis, a low density of small stomata (<13 mm^{-2}) and numerous sunken glandular hairs on both surfaces. Mesophyll is homogeneous with abundant extravascular fibre bundles. The root has a multilayered velamen with abundant tilosomes. Numerous idioblasts with raphides were observed in leaves, pseudobulbs and roots. The seeds are fusiform, with smooth surfaces and transverse folds. Some of these traits link *O. maculata* with terrestrial and epiphytic habits and with xerophytic habits, with humid and high light intensity and humid environments. This combination of traits might be a key factor behind the success and expansion of *O. maculata*. Nonetheless, a detailed characterization of the microhabitats occupied, demography, reproductive strategies and mycorrhizal associations will be essential for understanding the behaviour of this invasive species and, if necessary, designing strategies for its control.

ADDITIONAL KEYWORDS: anatomical adaptation – invasive plants – structural biology – structure/function relationships – terrestrial orchids.

INTRODUCTION

The adaptive responses of plants are expressed in morpho-anatomical and physiological strategies that modify the external morphology of the plant, the anatomy of cells, tissues and organs and the thresholds of diverse physiological parameters. These structural and physiological variations permit plants to survive and reproduce in a variety of environmental conditions and contribute to protection against stress and herbivore damage (Lambers, Stuart & Pons, 1998; Dickisson, 2000).

Particularly in the case of Orchidaceae, most reviews of the anatomy of groups are of a descriptive nature, and have focused upon the search for similarities or differences that contribute to the taxonomic determination and systematic of the group in question (Solereder & Meyer, 1930; Pridgeon & Stern, 1982; Stern *et al.*, 1993b; Kurzweil *et al.*, 1995; Stern, 1997; Holtzmeier, Stern & Whitten, 1998; Stern & Judd, 2001, 2002; Sandoval-Zapotitla *et al.*, 2003; Stern, Judd & Carlsward, 2004; Carlsward, Stern & Bytebier, 2006; Figueroa *et al.*, 2008; Sandoval-Zapotitla, Terrazas & Villaseñor, 2010; Aybeke, 2012; Pedroso-de-Moraes *et al.*, 2012). However, in some cases, anatomical characteristics have been analysed from an ecological-evolutive point of view, with the intention of recognizing the adaptive capacity of the family and to compare the adaptations to different environmental scenarios (Moreira & Isaias, 2008; Dugarte-Corredor & Luque-Arias, 2012; Moreira, Lemos-Filho & Isaias, 2013).

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Orchidaceae, with 25 000–30 000 species (Dressler, 1993, 2005), exhibit a high level of specialization and a great capacity for adaptation to a diversity of environments (Benzing, Ott & Friedman, 1982), which have contributed to the development of morphological, anatomical and physiological adaptations (Pabst & Dungs, 1975) and vegetative organization that vary between species (Dressler, 1993).

Despite the fact that the global compendium of weeds (www.hear.org/gcw/index.html) lists >90 species of orchids (Ackerman, 2007), little work has been done on the anatomy of these taxa. In general, studies on invasive orchids have concentrated on questions of distribution (Neto, Miranda & Cruz, 2011), modelling of ecological niches (Kolanowska, 2013; Kolanowska & Konowalik, 2014) or ecological interactions with native and naturalized organisms (De Long *et al.*, 2013; Recart, Ackerman & Cuevas, 2013; Ackerman *et al.*, 2014) or other invaders (Liu & Pemberton, 2010).

Oeceoclades maculata (Lindl.) Lindl. (Fig. 1A, B) is a terrestrial orchid, native to tropical Africa (Williamson, 2012; Romero-González, 2014), and is one of the most successful invasive organisms in tropical regions of the world, having colonized many Neotropical areas in the last 184 years (Adamowski, 1999; Acevedo-Rodríguez & Strong, 2012; GBIF, 2015). Few studies related to the anatomical structure of this species have been published. Silva *et al.* (2010a) described the macromorphology of the taxon, with emphasis on certain anatomical characteristics of the root. Nieto & Damon (2008) described the pollinaria and pollinia and Aguiar *et al.* (2012) described anatomical traits of the floral nectaries. Stern & Judd (2002) described roots, pseudobulbs and leaves of *Oeceoclades* Lindl., including *O. maculata* and *O. saundersiana* (Rchb.f.) Garay & P.Taylor.

Taking into account the wide distribution and rapid extension of this invasive orchid (Adamowski, 1999; Acevedo-Rodríguez & Strong, 2012; GBIF, 2015) an anatomical characterization of the vegetative organs, peduncles and seeds of individuals of *O. maculata* pertaining to different environmental conditions was carried out, with the objective of identifying characteristics with a possible ecological and/or physiological importance. This analysis might permit the definition of the adaptive strategies of *O. maculata* and the traits that confer advantages in its invasive behaviour.

MATERIAL AND METHODS

SAMPLING SITES

In 2014, individuals of *O. maculata* were collected in four ecosystems in three municipalities in the region of
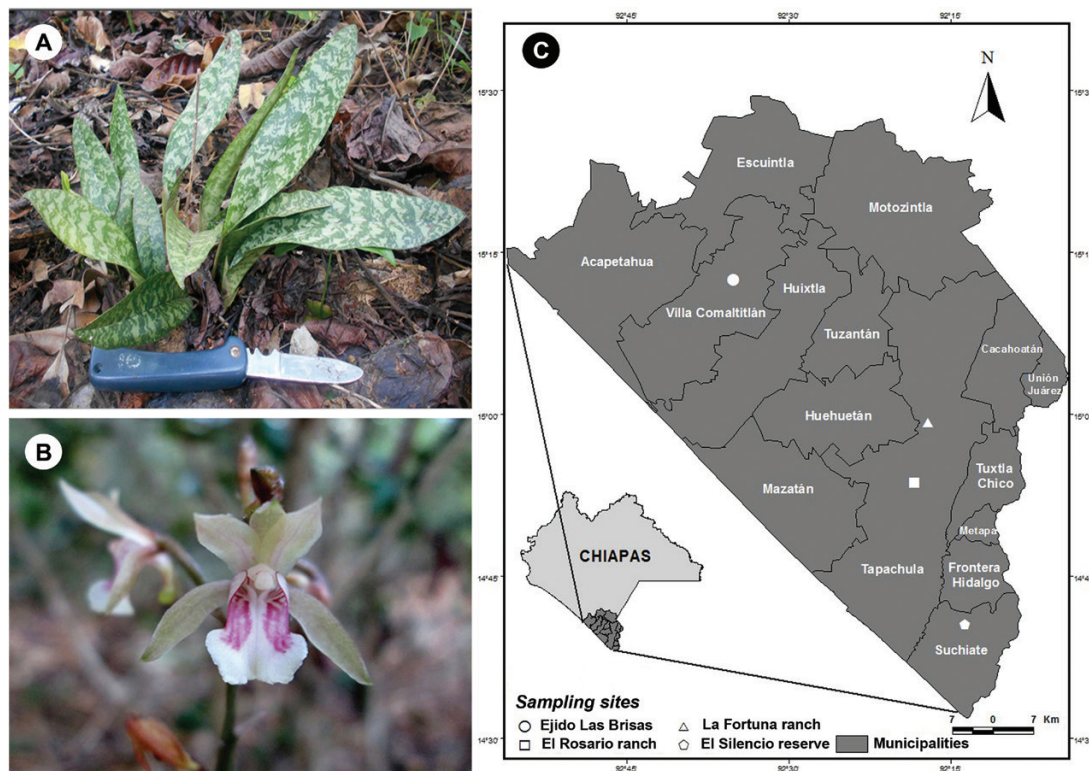

Figure 1. Adult plant of *Oeceoclades maculata* (A), flower (B) and location of the populations studied in Soconusco, Chiapas, Mexico (C).

Soconusco, Chiapas, in south-eastern Mexico (Table 1; Fig. 1C). The selection of the sites was based on the presence of *O. maculata* and the type of vegetation and management; sites that differed most from each other were selected in the hope of finding different anatomical responses in *O. maculata*.

ENVIRONMENTAL VARIABLES

Using a geographical information system (GIS), the geographical coordinates of each selected sampling site (locality) were used to find the corresponding values for mean annual temperature, annual precipitation, solar radiation and humidity (Table 2). The digital cartography consulted for mean annual temperature and precipitation was derived from Cuervo-Robayo *et al.* (2013), generated at high resolution for Mexico. For solar radiation, the data available for each season (spring, summer, autumn and winter) generated by Galindo, Castro & Valdés (1990a, b, c, d) were used, at a scale of 1:16 000 000. In the case of humidity the ranges of humidity generated by García (1990) were consulted at a scale of 1:4 000 000.

COLLECTION AND PROCESSING OF BIOLOGICAL MATERIAL

At each locality, 15 adult individuals of *O. maculata* (60 individuals in total) were collected. Each individual was labelled according to locality and samples of each structure (root, pseudobulb, leaf; 60 samples of each structure) were taken. From each of five individuals per locality a sample of peduncle (20 samples in total) was collected, and a single mature fruit was collected per locality to obtain samples of seeds. The fruits were wrapped in drying paper and stored in glass containers.

Root: a portion of root 10–15 cm long was cut from each individual. *Pseudobulb*: a transverse section (TS) was cut from the middle of a fully developed pseudobulb of each individual, measuring *c.* 1.0 cm in width. *Leaf*: a section from the middle of a fully developed leaf was taken from each individual, measuring *c.* 2.0 cm in width. *Peduncle*: a 2.0 cm section was selected from the middle zone of the peduncle of each individual. *Seeds:* 50 representative seeds were extracted from each fruit (200 seeds in total). The samples of roots, pseudobulbs, leaves and peduncles were fixed in FAA (0.5:0.5:9 parts commercial formalin, glacial acetic acid and ethanol) for 48 h and were then transferred to 70% ethanol (Johansen, 1940), before processing.

In the laboratory, the samples were placed onto polystyrene foam supports, on which the sections were made manually, using a razor blade. For the leaves, TS and longitudinal section (LS) were made and the adaxial and abaxial epidermises were separated from the sections using tweezers and the needles from insulin

Table 1. General characteristics of the localities in Soconusco, Chiapas, Mexico where populations of *Oeceoclades maculata* (Orchidacaeae) were studied

Locality	Ecosystem	Geographical coordinates	Elevation (m)
La Fortuna ranch	Shaded robusta coffee plantation (*Coffea canephora* Pierre ex A.Froehner)	14°59′12.1″N 0.92°17′06.5″W	356
Ejido Las Brisas	Shaded cocoa plantation (*Theobroma cacao* L.)	15° 12′26.82″N 092°35′3.78″W	40
El Silencio reserve	African palm plantation (*Elaeis guineensis* Jacq.)	14°40′35.34″N 092°13′39.54″W	32
El Rosario ranch	Secondary forest	14°53′41.3″N 0.92°18′23.0″W	133

Table 2. Environmental variables corresponding to the geographical coordinates of the populations of *Oeceoclades maculata* studied, in Soconusco, Chiapas, Mexico

Locality	Solar radiation (MJ/m³)				Humidity scale	Mean annual temperature (°C)	Mean annual precipitation (mm)
	Summer	Spring	Autumn	Winter			
La Fortuna ranch	18–19	21–22	18–19	18	Humid (m)	26.2	3302
Ejido Las Brisas	19	21–22	18–19	18	Humid (m)	27.4	3087
El Silencio reserve	18–19	21–22	18–19	18	Subhumid (w2)	27.3	1431
El Rosario ranch	18–19	21–22	18–19	18	Humid (m)	27.2	2282

Mean annual temperature and precipitation (Cuervo-Robayo *et al.*, 2013), solar radiation (Galindo *et al.*, 1990a, b, c, d), humidity (García, 1990).

syringes. Transverse and paradermal sections were made from the pseudobulbs, and TS and LS were made from the roots.

The sections of the different plant organs were observed using a light microscope (LM) and a scanning electron microscope (SEM). For observation with the LM, the samples were first bleached in commercial sodium hypochlorite for 3–5 min, washed with distilled water and placed into 70% alcohol. The sections were stained with safranin O alcoholic solution (Berlyn & Miksche, 1976; Kraus & Arduin, 1997) to facilitate the observation of sclerenchyma fibres and the cuticle; sections were also stained with Lugol's solution to confirm the presence of starch (Johansen, 1940). Mounted, semi-permanent preparations were made of all the samples observed, using water–glycerine and paraffin wax as the sealant. The preparations were photographed using transmitted light via the LM (Zeiss Axio Imager A1).

The sections and seeds of *O. maculata* were prepared for SEM observation by serial alcohol dehydration and they were then washed and dried to critical point (Critical Point Dryer: CO_2; SPI SUPPLIES; model SPI-DRY CPD). The samples were then mounted onto aluminium cylinders using double-sided, conductive carbon tape, covered with a layer of Au–Pd *c*. 20 nm thick using a gold–palladium metal depositor (DENTON VACCUM; model DESK II). The observations and photomicrographs were taken using an SEM (TOPCON; model SM-510).

Measurements of the structures were made on the photomicrographs using the programme FIJI (Schindelin *et al.*, 2012). For the root sections, the thickness of the velamen, the exodermis and the cortex were measured. The characteristics of the cells and other structures in TS and LS were described. For the pseudobulbs the width of the cuticle and epidermis were measured and those structures, with the parenchyma, were described from the TS. For leaves, the cuticle, epidermis and mesophyll were measured and described, with the characteristics of the vascular bundles, fibres and cells of the mesophyll, all derived from the TS. The presence of stegmata was confirmed from LS. The density of stomata and glandular hairs was determined and descriptions were made of the pattern of the stomata from the paradermal view, according to Metcalfe & Chalk (1988), and of the epicuticular wax, according to Wilkinson (1979). To determine densities, the stomata and glandular hairs were counted in three fields per sample, for each of the 30 samples of epidermis (15 adaxial, 15 abaxial) per locality (90 fields per locality), from which the area (in mm²) was determined and the number of stomata or glandular hairs per square millimetre was calculated. In the same way, the length and width of the stomata in the three fields per sample were also measured.

STATISTICAL ANALYSIS

Using the programme STATISTICA 8.0, non-parametric tests were performed to detect differences in the densities of the stomata between the adaxial and abaxial surfaces of the leaves, and between the different localities. To make comparisons between the superficies the Mann–Whitney *U*-test was used and to compare densities of stomata and glandular hairs between localities the Kruskal–Wallis test was applied. A pairwise Mann–Whitney *U*-test was used to determine the sites with maximum differences. In the case of the cuticle width, it was only possible to compare three of the four localities (Ejido Las Brisas, El Silencio reserve, El Rosario ranch), the data were logarithmically transformed and the analysis was carried out using an analysis of variance (ANOVA). Similarly, an ANOVA was applied to compare the thickness of the velamen in root samples from the four localities. For the analysis of the width of the cuticle and the velamen a *post hoc* Tukey test was applied to determine sites with maximum differences for the variables studied. The mean and standard deviation were applied as descriptive statistics.

RESULTS

LEAVES

Surface

The leaves of *O. maculata* are amphistomatous (Fig. 2A, B). The epidermal cells are generally polygonal (five- or six-sided; Fig. 2C) or rarely rectangular, with straight, occasionally curved, thickened anticlinal walls and with the same structure on both surfaces. The stomata are usually isolated, but are occasionally found in pairs or rarely in groups of three or four. For the stomata, 32% were anomocytic and surrounded by five or six cells (Fig. 2D), whereas 68% were tetracytic (Fig. 2E). The cuticular covering of the stomata was elliptical, with a slit-shaped opening (Fig. 2F). Stomatal density was greater on the abaxial surface (10.91 ± 2.08 mm^{-2}; Fig. 2A) than the adaxial one (3.29 ± 1.17 mm^{-2}; Fig. 2B). Similarly, the stomata were also larger on the abaxial surface (length: 34.37 ± 2.77 µm, width: 35.12 ± 3.49 µm), compared to adaxial one (length: 31.87 ± 2.93 µm, width: 31.07 ± 2.84 µm), as were the stomatal pores (abaxial diameter: 23.27 ± 2.37 µm, compared to adaxial diameter: 19.64 ± 2.73 µm). On both surfaces, abundant sunken, glandular hairs were observed (Fig. 2G), and, again, with a greater density on the abaxial than the adaxial surface (4.03 ± 1.22 compared to 1.61 ± 0.68 mm^{-2}).

Transverse sections

The cuticle of the leaves had a smooth surface (Fig. 2J) and was thin to slightly thickened (3.87 ± 1.19 µm on
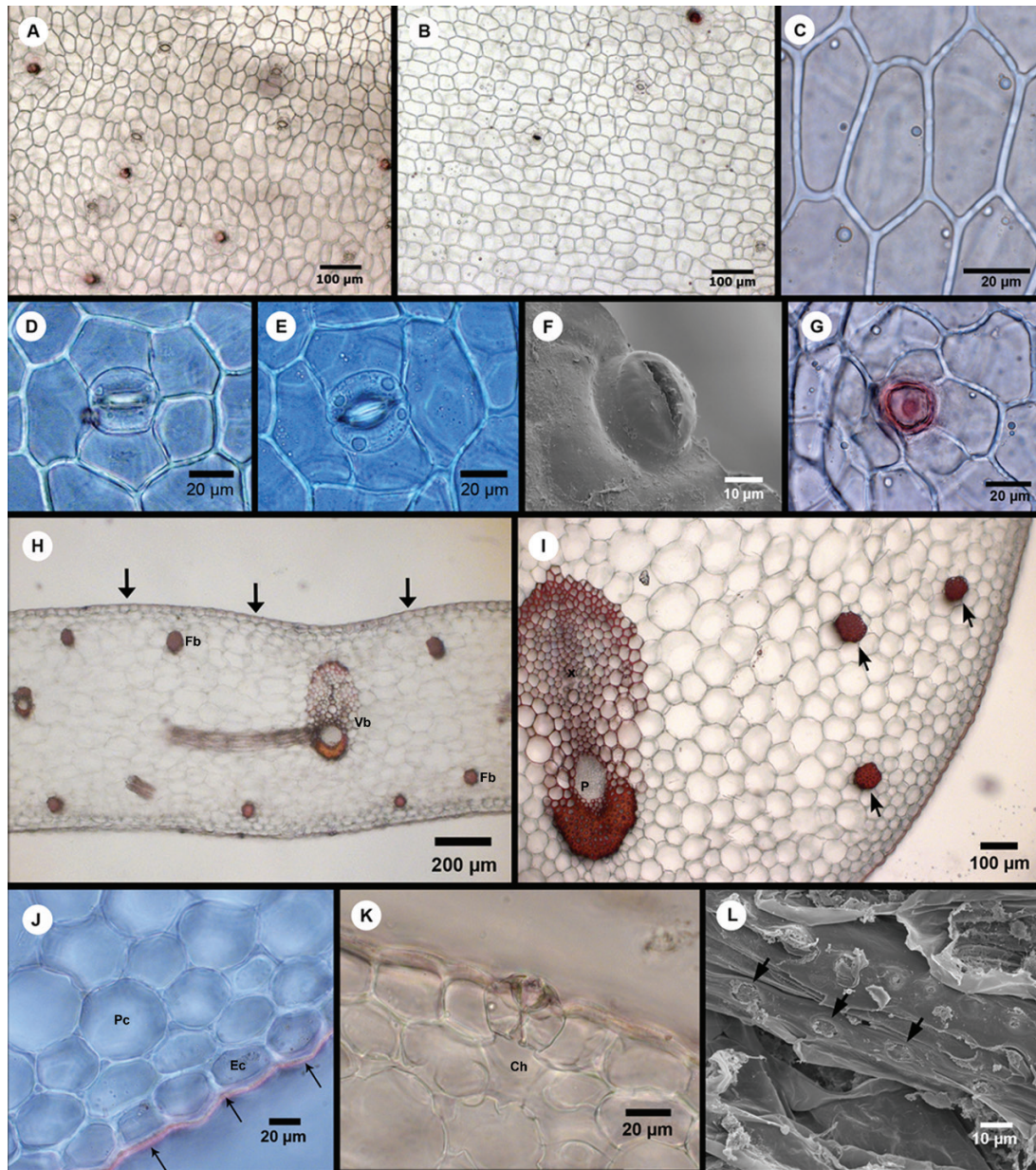
Figure 2. Anatomy of the leaves of *Oeceoclades maculata* in Soconusco, Chiapas, Mexico. (A–G) Paradermal view. (H–K) Transverse section. (A) Abaxial surface. (B) Adaxial surface. (C) Pentagonal and hexagonal epidermal cells. (D) Anomocytic stomata. (E) Tetracytic stomata. (F) SEM view of the ellipsoid cuticular covering and the slit-shaped stomatal aperture. (G) Sunken glandular hair. (H) Homogeneous mesophyll, vascular bundles in a line and extravascular fibres close to the epidermis, adaxial surface (arrowheads). (I) Detail of the collateral vascular bundle with sclerenchyma fibres at the poles and extravascular fibre bundles (arrowheads). (J) Uniseriate epidermis and smooth cuticle (arrowheads). (K) Stomata and substomatal chamber. (L) SEM view of a longitudinal section showing stegmata (arrowheads) associated with extravascular fibre bundles. Collateral vascular bundle (Vb), extravascular fibre bundles (Fb), xylem (X), phloem (P), parenchyma cell (Pc), epidermal cell (Ec), substomatal chamber (Ch).

the adaxial surface and 3.15 ± 0.87 μm on the abaxial surface). On both surfaces the epidermal cells ranged from rectangular to square (Fig. 2J) were smaller than the surrounding cells and lateral and periclinal walls adjacent to the cuticle were all thickened. The stomata and the epidermal cells were at the same level and the substomatic chambers were similar in size, or slightly larger than the stomata, but were not larger than the

adjacent cells of the mesophyll (Fig. 2K). The hypodermis consisted of a single layer of cells only slightly differentiated from the surrounding tissue.

Abundant bundles of sclerenchyma fibres were observed in the mesophyll, either accompanying vascular bundles or solitary and close to the epidermis on both surfaces (Fig. 2H, I). The mesophyll was generally homogeneous (Fig. 2H), with an average width of 773.54 ± 305.11 µm, only differentiated from the adaxial part of the central vein where the cells close to the adaxial epidermis were seen to be longer than the cells of the rest of the mesophyll. The cells of the mesophyll had thin walls and were closely packed together. The cells ranged from polygonal to circular and were variable in size, being smaller in areas close to the abaxial and adaxial epidermis, and larger in the centre of the mesophyll. The intercellular spaces were small, and triangular or irregular.

Collateral vascular bundles were situated in a single row along the longitudinal axis of the leaf (vascular bundles in the central area were most developed; Fig. 2I), with various layers of sclerenchyma cells with thickened walls at both poles and laterally joined by cells with thinner walls. Xylem and phloem were embedded in parenchyma cells with thickened walls. Stegmata (Fig. 2L) were observed from the longitudinal view, and were associated with the vascular bundles and extravascular fibres. Circular to oval idioblasts with thin walls and with bundles of raphides inside were frequent between the cells of the mesophyll and were most frequent near the epidermis and along the leaf margins.

Considering the adaxial and abaxial surfaces combined, significant differences were found between the densities of the stomata (Kruskal–Wallis; $H_{(3, 506)} = 10.23$, $P = 0.016$) and the sunken glandular hairs (Kruskal–Wallis; $H_{(3, 506)} = 18.71$, $P < 0.001$) in relation to the four localities. The greatest difference in the stomatal density was observed between Ejido Las Brisas and the El Silencio reserve (Mann–Whitney $U = 11353.5$, $P = 0.002$) with the lowest and highest densities, respectively. The lower density of sunken glandular hairs in Ejido Las Brisas differed significantly from the other three localities [La Fortuna ranch (Mann–Whitney $U = 4276.5$, $P = 0.005$); El Silencio reserve (Mann–Whitney $U = 10776.5$, $P < 0.001$); El Rosario ranch (Mann–Whitney $U = 4124.5$, $P < 0.001$)]. The thickness of the cuticle differed significantly between the three localities studied ($F_{(2, 277)} = 13.46$, $P < 0.001$), with *O. maculata* plants from El Rosario ranch having the thickest cuticles (Tukey test, $P < 0.05$).

PSEUDOBULBS

The cuticle was thin and smooth (Fig. 3A), with 2.83 ± 1.18 µm of thickness. The epidermis had a single layer of cells with undulating walls in a paradermal view (Fig. 3C), and the cells were rectangular to square, as seen from the TS (Fig. 3A), and smaller in size than the surrounding cells, with a width of 26.73 ± 7.03 µm. The external walls of the epidermal cells were slightly thickened. Situated immediately beneath the epidermis was the hypodermis with a single layer of polygonal to rectangular cells which were slightly larger than the epidermal cells. The parenchyma cells were isodiametric, with thin walls (Fig. 3D), and the few intercellular spaces were triangular to irregular. Abundant circular to oval idioblasts were observed, with bundles of raphides inside, mostly situated close to the epidermis (Fig. 3E). Abundant vascular bundles were embedded in the storage tissue (Fig. 3B), at the phloem pole with abundant sclerenchyma fibres (Fig. 3D).

ROOTS

A single type of root was observed, which appeared to perform all the functions of support, absorption and storage of nutrients. The velamen had 7–14 layers of cells, with an average thickness of 412.54 ± 134.77 µm, which varied according to the dimensions of the root (Fig. 4A, B). The roots showed crests or undulations in certain areas (Fig. 4A) and had few root hairs on the surface (Fig. 4B). The cells adjacent to the exodermis were the smallest, and polygonal or slightly rectangular as seen from the TS. The cells furthest away from the exodermis were the largest and increasingly elongated towards the margin of the velamen. All the cells of the velamen showed thickening of the cell walls, in the form of bands, more or less parallel, sometimes branched and crossing (Fig. 4F). The exodermis was uniseriate (Fig. 4B), with more or less oval cells, as seen by TS (Fig. 4D) and elongated in LS. The walls of the exodermal cells were slightly thickened in a U shape, mainly in the region in contact with the velamen (Fig. 4D). Abundant passage cells were observed (Fig. 4D, E) distributed intermittently between the cells of the exodermis. In the region of the velamen adjacent to the passage cells, webbed tilosomes were observed (Fig. 4E). The cortex (Fig. 4A) consisted of 12–20 layers of cells with a thickness of 1221.50 ± 288.27 µm, depending upon the thickness of the root. The cells of the parenchyma of the cortex were mostly circular to polygonal and isodiametric, with thin walls. The size of the cells varied according to the position and cells closest to the exodermis and the vascular cylinder tended to be smaller. The intercellular spaces were small and mostly triangular shaped. In some of the parenchyma cells pelotons composed by hyphae of endomycorrhizal fungi were observed in various stages of digestion (Fig. 4H) and abundant starch grains were observed in the reserve cells (Fig. 4I). Throughout the length of the root, in the parenchyma, abundant idioblasts with raphides bundled inside were observed, which were more abundant towards the root apex. The
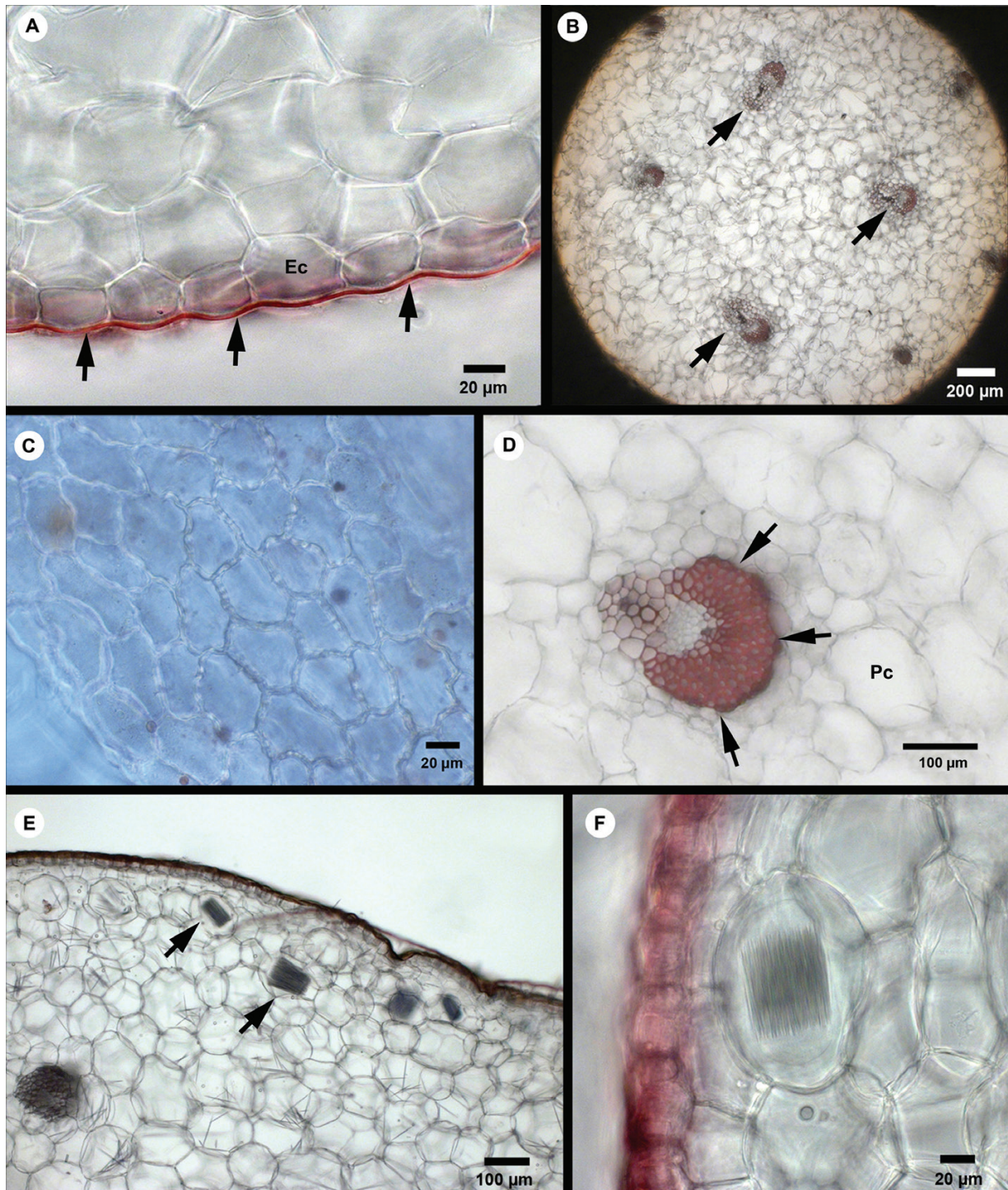
Figure 3. Anatomy of the pseudobulb of *Oeceoclades maculata* in Soconusco, Chiapas, Mexico. (A, B, D, E, F) Transverse section. (C) Paradermal view. (A) Epidermis and cuticle (arrowheads). (B) Parenchyma with free, collateral vascular bundles (arrowheads). (C) Epidermal cells with sinuous (undulate) anticlinal walls. (D) Detail of a collateral vascular bundle with thickened sclerenchyma fibres (arrowheads), mostly at the phloem pole. (E) Idioblasts (arrowheads) close to the epidermis. (F) Oval idioblast with a bundle of raphides inside. Epidermal cell (Ec), parenchyma cell (Pc).
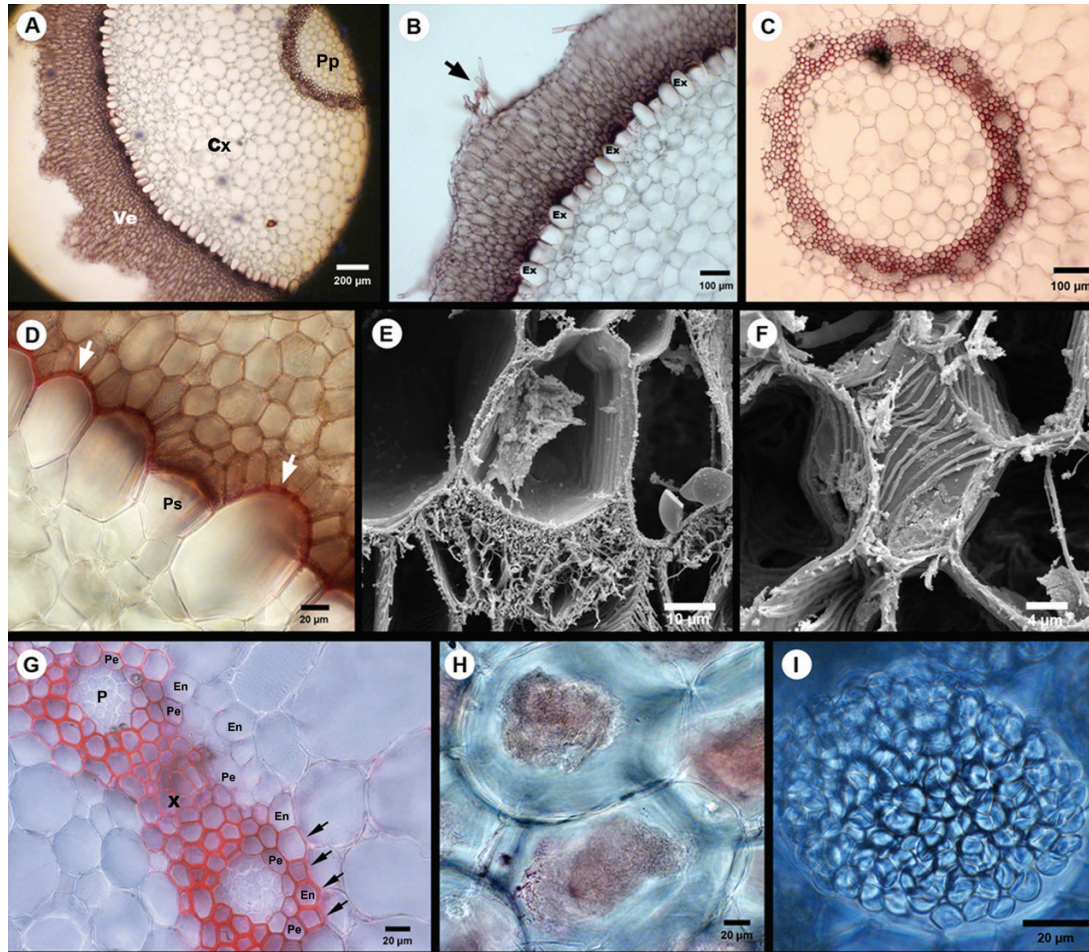
Figure 4. Anatomy of the root of *Oeceoclades maculata* in Soconusco, Chiapas, Mexico. (A) Transverse section. (B) Detail of the velamen with root hairs (arrowhead). (C) Vascular cylinder and parenchymatous pith. (D) Exodermis with thickening (arrowheads) at the point of contact with the velamen and passage cell. (E) SEM view of the passage cell and adjacent webbed tilosome. (F) Thickening of the cell walls of the velamen. (G) Endodermis with O-shaped thickening (arrowheads) and poles of xylem and phloem. (H) Partially digested pelotons. (I) Starch grains in reserve cell within the parenchyma. Velamen (Ve), cortex (Cx), parenchymatous pith (Pp), exodermis (Ex), passage cell (Ps), endodermis (En), phloem (P), xylem (X), pericycle (Pe).

endodermis consisted of a single layer of cells, in which the Casparian strips were not clearly distinguishable; these cells were generally isodiametric with thickening in the shape of an 'O' in areas opposite the phloem, and with thin walls where they faced the xylem (Fig. 4G). The pericycle had a single layer of polygonal and isodiametric cells, with thickened walls in the shape of an 'O' in areas opposite the phloem, and with thin walls where they faced the xylem. The vascular cylinder consisted of 10–26 arches depending upon the diameter of the root (Fig. 4C). The xylem elements were arranged radially, alternating with oval or circular patches of phloem cells. Both vascular elements were surrounded by a sheath of sclerenchyma fibres. The pith consisted of circular to polygonal parenchyma cells, with thin walls, irregular or triangular shaped intercellular spaces (Fig. 4C),

and in many cases with abundant starch grains inside. The thickness of the velamen was significantly different among the four localities studied ($F_{(3, 226)} = 7.97$, $P < 0.01$), with La Fortuna ranch showing the greatest difference, with the thickest velamen (Tukey test, $P < 0.05$).

PEDUNCLE

This structure was seen as circular in TS (Fig. 5A), with a diameter of 2512.38 ± 63.50 µm. The cuticle was thin to moderately thick, 3.29 ± 1.31 µm, and smooth. The epidermis was thin, measuring 19.39 ± 3.07 µm, with a single layer of cells each with rectangular or oval shape (Fig. 5B). The thin cortex measured 134.85 ± 30.01 µm and consisted of four to seven layers
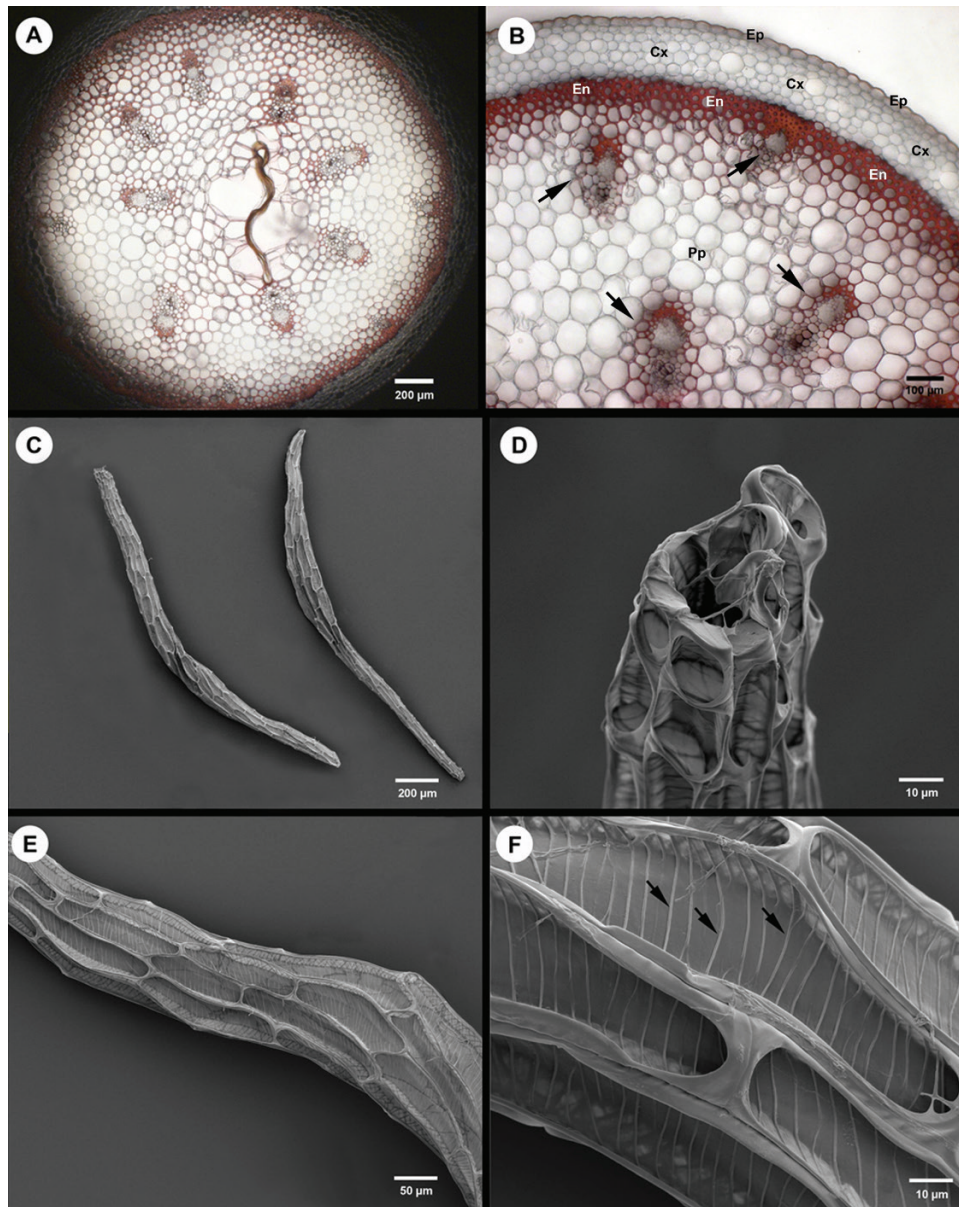
Figure 5. Anatomy of the peduncle and the seeds of *Oeceoclades maculata* in Soconusco, Chiapas, Mexico. Transverse section of the peduncle as seen with light microscope (A, B). SEM view of seeds (C–F). (A) Collapsed parenchymatous pith with vascular bundles arranged in a ring. (B) Detail of the peduncle with collateral vascular bundles (arrowheads). (C) Fusiform seeds. (D) Chalazal pole. (E) Cells of the testa. (F) Detail of testa cells with transverse thickening of the periclinal walls (arrowheads). Parenchymatous pith (Pp), epidermis (Ep), cortex (Cx), endodermis with thickening of the sclerenchyma (En).

of cells (Fig. 5B). Those cells were spherical or polygonal, with thin walls and irregular or triangular shaped intercellular spaces. The endodermis had a thickened layer of three to six sclerenchyma cells into which some of the vascular bundles were embedded (Fig. 5B). The vascular cylinder consisted of 6–15 collateral vascular bundles, some of which were embedded in the ring of sclerenchyma and others which were embedded in the parenchyma of the pith, forming two concentric rings (Fig. 5A, B). The vascular bundles were thickened with

sclerenchyma cells towards the poles. The parenchyma cells of the pith were polygonal to circular, with thin walls and irregular or triangular shaped intercellular spaces. In some cases, the central area of the pith was seen to be collapsed (Fig. 5A).

SEEDS

The seeds were fusiform (Fig. 5C), measuring on average 1699.22 ± 194.24 µm in length and 138.21 ± 19.47 µm in

width. The chalazal pole was blunt tipped (Fig. 5D). The longitudinal axis consisted of an average of 16.7 ± 1.4 cells. The cells of the surface of the seeds were elongated to rectangular (Fig. 5E), with an average length of 120.36 ± 48.46 μm and width of 22.51 ± 8.42 μm. The anticlinal walls were raised (height 10.01 ± 2.88 μm), straight and without ornamentation. The periclinal walls had oblique, thickened bands throughout their surface (Fig. 5F). At the points of union between the cells, the anticlinal walls were seen to be curved towards the interior forming a smooth flange (Fig. 5F).

DISCUSSION

The monk orchid (*O. maculata*) is one of the most successful invasive organisms in tropical regions of the world (Adamowski, 1999). Our results indicate that this species display some common anatomical traits previously reported in Orchidaceae, but at the same time this species has characteristics reported for both terrestrial and epiphytic habits and for orchid species adapted to humid, shaded environments and also to xerophytic conditions with abundant light intensity. In the following section, we discuss these features and some other anatomical traits that could contribute to the fitness of this species further.

In general, orchids have hypostomatous leaves (Withner, Nelson & Wejksnora, 1974; Singh, 1981; Zanenga-Godoy & Costa, 2003; Stern *et al.*, 2004; Silva *et al.*, 2006; Kumar & Krishnaswamy, 2014). However, the leaves of *O. maculata* are amphistomatous as pointed by Stern & Judd (2002), a characteristic typical of the leaves of plants adapted to dry environments (Fahn & Cutler, 1992) and, in combination with a homogeneous mesophyll, are considered to be adaptations for the epiphytic habit (Colleta & Silva, 2008; Bercu, Bavaru & Broasca, 2011). The localities selected for this study all fell within the range of humid to subhumid (Table 2), and *O. maculata* is considered a terrestrial and only occasionally epiphytic species (Romero-González, 2014). The amphistomatous nature of the leaves could be the result of various interplaying factors, such as the relatively vertical orientation of the leaves (Tominsky, 1905) and the relative thickness of the leaves, which in combination with variegation are characteristics associated with crassulacean acid metabolism (CAM; Rasmussen, 1987; Bone *et al.*, 2015). Furthermore, it could also be a consequence of adaptive radiation from the terrestrial to the epiphytic habit, taking into account that the ancestors of tropical orchids were terrestrial (Silvera *et al.*, 2009) and that the plesiomorphic state of subfamily Epidendroideae, to which *O. maculata* belongs, is terrestrial (Freudenstein & Chase, 2015).

The stomatal density of the leaves of *O. maculata* is low and even lower than that reported for other species of terrestrial orchids (Sgarbi & Del Prete, 2005; Dugarte-Corredor & Luque-Arias, 2012; Franco, 2013), including the invasive *Spathoglottis plicata* Blume (Mulgaonkar, 2011). In addition, it is lower than that reported for epiphytic orchid species (Yukawa *et al.*, 1992; Moreira *et al.*, 2009; Rosa-Manzano *et al.*, 2014). Stomatal density has been shown to be positively related to light intensity (Cyge, 1930; Schoch, Zinsou & Sibi, 1980) and negatively related to shade levels (Lake *et al.*, 2001). It is a characteristic reported as an adaptation to shaded conditions (Valladares & Niinemets, 2008; Moreira *et al.*, 2009). On the other hand, the reduction in stomatal density could function as a mechanism for the control of transpiration (Colmenares-Arteaga, Rada & Luque, 2005) and could help to maintain the water balance during unfavourable seasons and therefore reduce the seasonal physiological variation (Rosa-Manzano *et al.*, 2014).

Other features of leaf anatomy of *O. maculata* have been previously reported in Orchidaceae such as the anomocytic and abundant tetracytic stomata (Solereder & Meyer, 1930; Rasmussen, 1987; Stern & Judd, 2002; Silva & Milaneze-Gutierre, 2004; Colleta & Silva, 2008). The presence of collateral vascular bundles and homogeneous mesophyll (Oliveira & Sajo, 1999a; Stern & Judd, 2002; Zanenga-Godoy & Costa, 2003; Stern *et al.*, 2004; Silva *et al.*, 2006; Colleta & Silva, 2008; Aybeke, 2012; De Cássia, de Barros & das Graças, 2015). The smooth and thin to moderately thick cuticle (Stern *et al.*, 1993b; Morris, Stern & Judd, 1996; Barthlott *et al.*, 1998) which is slightly thicker on the adaxial surface (Holtzmeier *et al.*, 1998; Arévalo, Figueroa & Madriñán, 2011).

The stomata in *O. maculata* are smaller than those found in other terrestrial orchid species (Stern & Judd, 2002; Sgarbi & Del Prete, 2005) and even smaller than those in some epiphytic species of Cymbidieae (Stern & Judd, 2002), the tribe to which *O. maculata* belongs. They are also smaller than those of *O. saundersiana* (Stern & Judd, 2002) the only other species of the genus with a partial anatomical description. Small stomata are more related to epiphytic orchid species (Solereder & Meyer, 1930; Rasmussen, 1987; Paek & Jun, 1995). They enable plants to respond more quickly to environmental changes or to the decrease in leaf water potential and they also promote greater diffusive conductance under favourable conditions (Aasamaa, Sober & Rahi, 2001; Drake *et al.*, 2013). Larger stomata are slower to close than smaller ones and they increase the possibility of hydraulic dysfunction in dry conditions (Aasamaa *et al.*, 2001). Therefore, especially under dry conditions the small stomata of *O. maculata* could represent an adaptive advantage.

The presence of stomata at the same level as the surrounding epidermal cells and with suprastomatic chambers is a common characteristic of epiphytic and xerophytic orchids (Rosso, 1966; Oliveira & Sajo, 1999a; Zanenga-Godoy & Costa, 2003; Stern *et al.*, 2004; Silva *et al.*, 2006; Dugarte-Corredor & Luque-Arias, 2012). These structures form an air-filled chamber that reduces the rate of transpiration (Rasmussen, 1987) and, combined with a wider mesophyll, can be considered a response to high light intensity (Moreira *et al.*, 2013). The extravascular bundles in the mesophyll and the groups of sclerenchyma fibres that form a cap at both poles of the vascular bundles (phloem and xylem) have been previously reported (Shushan, 1959; Holtzmeier *et al.*, 1998; Stern & Judd, 2002; Silva & Milaneze-Gutierre, 2004; Silva *et al.*, 2006; Dettke *et al.*, 2008). However, these structures are more frequent in orchid species adapted to xerophytic habitats (Withner *et al.*, 1974; Rudall, 1986), probably related to mechanical resistance to dehydration (Bonates, 1993; Oliveira & Sajo, 1999a; Stern *et al.*, 2004). On the other hand, the presence of well-developed stomatal ledges that form a suprastomatic chamber has been reported for terrestrial orchids growing in temperate climates (Ziegenspeek, 1936) and in orchids adapted to swamps. In the latter case, these structures were interpreted as a protection of stomatal pores from blockage by the surrounding water (Rasmussen, 1987).

The sunken glandular hairs present on both surfaces (adaxial and abaxial) of the leaves of *O. maculata* have been described previously for this species (Stern & Judd 2002), also in the context of spiranthoids (Stern *et al.*, 1993b) and epidendroids (Pridgeon & Williams, 1979; Pridgeon, 1981, 1982; Benzing & Pridgeon, 1983; Holtzmeier *et al.*, 1998). They have been also classified as glands or cells that secrete wax (Ferry, 2008; Nengpilhing *et al.*, 2015). In other invasive orchids such as *S. plicata* and *Arundina graminifolia* (D.Don) Hochr., trichomes of glandular nature have also been found (Mulgaonkar, 2011; Sulistiarini & Tihurua, 2012). The role of these glandular trichomes is not yet fully understood. Pridgeon & Williams (1979) noted their possible function as active hydathodes or water glands. However, this hypothesis was rejected by Pridgeon (1981), who, on the basis of morphological comparisons and staining behaviour, proposed that the sunken glandular hairs were primarily absorptive and functionally similar to tillandsioid scales in Bromeliaceae. Later Benzing & Pridgeon (1983) showed that these glandular hairs do not function as significant absorption agents and may secrete mucilage instead, perhaps facilitating unfolding of the lamina. However, this function is not yet demonstrated, at least for these kind of sunken glandular hairs. Colleters are finger-like trichomes, composed of two uniseriated cells, that produce mucilage with lipophilic and proteinic compounds; indicating the involvement of these hairs with the protection of meristematic regions in vegetative and reproductive organs (Leitão & Cortelazzo, 2008; Mayer, Cardoso-Gustavson & Appezzato-da-Glória, 2011). Nevertheless, the sunken glandular hairs described in this study structurally differ from colleters and might have a different function. On the other hand, glandular trichomes have received considerable attention for their capacity to synthesize, store and secrete secondary metabolites that help to repel or kill pests and diseases, to reduce herbivory and to protect plants against other abiotic challenges (Levin, 1973; Dell & McComb, 1978; Wagner, 1991). Bearing in mind that some secondary metabolites such as alkaloids can be produced in orchids (Lüning 1964, 1967, 1974; Slaytor 1977) and that leaves of *O. maculata* showed few signs of pest damage, we propose that another function of the sunken glandular hair might be the production and secretion of secondary metabolites to protect leaves against different threats. However, this hypothesis needs to be tested.

Stegmata are structures that have previously been found in Orchidaceae (Sandoval-Zapotitla, 1993; Sandoval-Zapotitla & Terrazas, 2001; Stern & Judd, 2002; Stern & Carlsward, 2006; Sandoval-Zapotitla *et al.*, 2010). Notwithstanding, the microclimatic conditions necessary, the mechanisms by which silica dioxide is fixed and stegmata are formed in orchids and the adaptive advantages of these structures for the leaves, particularly in the case of stegmata associated with fibres are unknown (Sandoval-Zapotitla *et al.*, 2010). Prychid, Rudall & Gregory (2004) proposed that the silica that makes up stegmata could contribute to the rigidity of the organ concerned and could also be related to defence against herbivory and infection by microorganisms. Other authors have suggested that the presence of stegmata in the epidermis of some epiphytic orchids could be associated with the xerophytic condition (Zanenga-Godoy & Costa, 2003). In the case of *O. maculata*, pest or disease damage is rarely seen and the presence of stegmata could be a contributory factor.

Pseudobulbs are considered a characteristic of epiphytic orchids and secondary terrestrial orchids (Zimmerman, 1990; Hew, Koh & Khoo, 1998; Kozhevnikova & Vinogradova, 1999; Stancato, Mazzafera & Buckeridge, 2001). They are reserve organs capable of storing minerals (Zimmerman, 1990), water (Zimmerman, 1990; Stancato *et al.*, 2001) and carbohydrates (Zimmerman, 1990; Hew *et al.*, 1998; Stancato *et al.*, 2001). The structural integration of pseudobulbs is relatively similar across Orchidaceae (Stern *et al.*, 2004).

The velamen of *O. maculata* is classified as non-specific according to Porembski & Barthlott (1988) and the thickening of the cell walls is a combination

of groups I and II according to the classification for the striations of the wall of velamen cells made by Sanford & Adanlawo (1973). In orchid roots, velamen is usually associated with the epiphytic habit (Engard, 1944; Dycus & Knudson, 1957; Pedroso-de-Moraes *et al.*, 2012), but it has also been recorded in terrestrial species (Porembski & Barthlott, 1988; Stern et al., 1993a, b; Kurzweil *et al.*, 1995; Stern & Judd, 2002) and is absent in some taxa (Singh, 1986). The presence of a velamen has also been recorded in other monocotyledons such as Araceae, Liliaceae, Dioscoreaceae, Taccaceae, Amaryllidaceae (including *Agapanthus* L'Hérit.), Asparagaceae and Commelinaceae (Dahlgren & Clifford, 1982; Cutler *et al.*, 2008). Various functions have been attributed to this structure, including increasing access to mineral solutions and the absorption of nutrients (Benzing *et al.*, 1982; Zotz & Winkler, 2013), a reduction in transpiration rates, reflectance of infrared radiation, mechanical protection (Noel, 1974; Benzing *et al.*, 1982; Pridgeon, 1986), facilitating the exchange of oxygen and carbon dioxide with the atmosphere and constitutes a water reserve (Dycus & Knudson, 1957; Sanford & Adanlawo, 1973; Dressler, 1993). The size of the velamen could be associated with environmental conditions such as temperature and water availability (Sanford & Adanlawo, 1973). The number of rows of cells in the velamen of *O. maculata* (7–14) contrasts with other terrestrial orchids, in which one to eight rows have been reported (Figueroa *et al.*, 2008; Silva, Meira & Azevedo, 2010b). The presence of a multiple-layered velamen has been reported as a characteristic of xerophytic, terrestrial orchids, adapted to dry environments with abundant solar radiation (Silva *et al.*, 2006, 2010b). However, in this study, the four selected localities do not conform to those characteristics, all having average annual precipitation >1000 mm (Table 2). The sinuous projections observed in the velamen of *O. maculata* have been reported in species with a single-layered velamen, where it is assumed to increase the area capable of absorbing water and mineral salts (Silva *et al.*, 2010b).

The presence of tilosomes are usually associated with the epiphytic habit in orchids (Benzing *et al.*, 1983; Pridgeon, Stern & Benzing, 1983; Porembski & Barthlott, 1988), but this has also been reported for terrestrial species (Benzing *et al.*, 1982; Figueroa *et al.*, 2008; De Cássia *et al.*, 2015), including *O. maculata* (Stern & Judd, 2002). Although the functions of tilosomes have not been fully defined (Pridgeon *et al.*, 1983), in the case of *O. maculata* these structures are abundant and could be involved in the absorption of water and minerals solutes, the modulation of moisture exchange (Engard, 1944; Benzing *et al.*, 1982; Pridgeon *et al.*, 1983; Pridgeon, 1987) or could participate in the prevention of the entry of pathogens (Holtzmeier *et al.*, 1998).

Other anatomical traits previously reported for roots of Orchidaceae and that were found in *O. maculata* are a single-layered exodermis with passage cells (Withner *et al.*, 1974; Benzing *et al.*, 1982, 1983; Pridgeon & Stern, 1982; Pridgeon *et al.*, 1983; Stern, 1997; Stern & Whitten, 1998; Stern & Judd, 2001; Stern *et al.*, 2004); the greater thickness of the parenchyma of the cortex than the parenchyma of the pith (Moreira & Isaias, 2008); the pith, with parenchyma cells with thin cell walls (Stern *et al.*, 1993b; Figueroa *et al.*, 2008; Moreira & Isaias, 2008; Dugarte-Corredor & Luque-Arias, 2012); the accumulation of starch in the cortex and pith (Zotz, 1999; Silva *et al.*, 2006; Moreira *et al.*, 2009); both the thickening of the endodermis and the delimitation of the vascular cylinder by a single-layered pericycle (Stern & Judd, 2002; Stern *et al.*, 2004; Carlsward *et al.*, 2006; Moreira & Isaias, 2008; Pridgeon *et al.*, 2009; Pedroso-de-Moraes *et al.*, 2012; Moreira *et al.*, 2013); and the variations in the number of poles of the xylem, when comparing the roots of the same species and also in different sections of the same root (Rosso, 1966; Singh, 1986; Fahn, 1990; Moreira *et al.*, 2013).

Pelotons formed by mycorrhizal fungi represent an important nutritional source for orchids (Lesica & Antibus, 1990; Senthilkumar *et al.*, 2000b). In Orchidaceae, the association with mycorrhizal fungi is vital for seed germination and subsequent differentiation and the establishment of the young plants (Arditti, 1992; Jersáková & Malinová, 2007; Chung, Nason & Chung, 2011). Many species maintain this association throughout their life, whereas in some cases the adult plant may become independent of the fungi (Arditti, 1967; Sanford, 1974).

Passage cells are abundant in *O. maculata*. According to some authors, these kind of cells are fundamental for the entry of water and mineral salts to the root cortex (Benzing et al., 1982, 1983; Fahn, 1990; Evert, 2006). Furthermore, the emission of signals for the attraction of endophytic fungi, and facilitating and controlling their passing through the cortex, is a secondary function also attributed to the passage cells (Peterson & Enstone, 1996; Senthilkumar *et al.*, 2000a; Chomicki, Bidel & Jay-Allemand, 2014). In addition, it is have been suggested that the distribution and density of passage cells define a strategy for controlling the extent and location of fungal invasion (Chomicki *et al.*, 2014). *Oeceoclades maculata* is a species that has a certain plasticity when it comes to establishing mycorrhizal associations, including reports of associations with *Epulorhiza repens* (Bernard) Moore, *Psathyrella candolleana* (Fr.) R.Maire, and other fungi taxa of the genera *Ceratobasidium* D.P.Rogers, *Tulasnella* J.Schröt. and *Fomitopsis* P.Karst. (Pereira *et al.*, 2005; Garriga *et al.*, 2009), in contrast to other terrestrial orchid genera such as *Cypripedium* L., for which a

marked specificity has been reported (Shefferson *et al.*, 2005). The fungi that have so far been reported associated with *O. maculata* have a wide distribution around the world (Roberts, 1999; Piepenbring, 2008), which is another factor that could explain the extended geographical distribution of this orchid (Pereira *et al.*, 2005; Garriga *et al.*, 2009). On the other hand, Bayman *et al.* (2016) find that *O. maculata* appears to be highly specific for fungi during seed germination in vitro, which occurs only in the presence of *Psathyrella*, but at the same time, it is unusually promiscuous as adult plants. These authors suggested that the mycorrhizal associations with *Psathyrella* and with other saprotrophic fungi, which have been only reported from mycoheterotrophic orchids, may partly explain the success of *O. maculata*.

The general structure of the peduncle of *O. maculata* is similar to that of the terrestrial orchids *Aa paleacea* (Kunth) Rchb.f., *Myrosmodes paludosa* (Rchb.f.) Garay, *Pterichis multiflora* (Lindl.) Schltr. and *Malaxis termensis* (Kraenzl.) Schweinf. (Dugarte-Corredor & Luque-Arias, 2012; Franco, 2013), but differs in having a circular, instead of an elliptical contour.

Raphides are the most common mineral inclusions in monocotyledons (Prychid & Rudall, 1999) and are commonly found in orchids (Pridgeon, 1982; Stern *et al.*, 1993b; Oliveira & Sajo, 1999a; Zanenga-Godoy & Costa, 2003; Silva *et al.*, 2006; Sandoval-Zapotitla *et al.*, 2010; Bercu *et al.*, 2011), particularly in epiphytes (Stern *et al.*, 2004). These structures may be involved in defence, mechanical support, ionic exchange, osmotic control and regulation of the levels of calcium in the phloem (Franceschi & Horner, 1980; Bonates, 1993; Mauseth, 1995; Pandey, 2001; Paiva & Machado, 2005). The abundance of bundles of raphides found in areas close to the epidermis of the leaves, pseudobulbs and in the exodermis and tips of the root of *O. maculata* suggest the functions of mechanical support and defence.

Fusiform-shaped seeds, as seen in *O. maculata*, are a common trait of orchids (Beer, 1863; Arditti, 1967, 1979; Barthlott, 1976). The variation in the length of the seed, the number of cells that make up the testa and the folds in the periclinal walls are similar to those found in the terrestrial orchid genera *Goodyera* R.Br., *Piperia* Rydb., *Platanthera* Rich. and *Spiranthes* Rich and the width of the cells falls within the range reported for these genera (Healey, Michaud & Arditti, 1980). However, the seeds of *O. maculata* are longer. Considering the functional correlation between the morphology and the aerodynamics and capacity for water absorption of the seeds (Senghas *et al.*, 1974; Barthlott, 1976; Arditti, 1979; Arditti, Michaud & Healey, 1980), the greater length of the testa could result in an increase of the amount of air within the seed which could then increase the air buoyancy and

facilitate dispersion (Barthlott, 1976; Healey *et al.*, 1980). Supporting this idea, it is been suggested that success of the colonization events of *O. maculata* results of a more effective seed dispersion (Ueno *et al.*, 2015).

Despite the fact that, in general, macro-environmental conditions were similar, we found differences in stomatal and sunken glandular hair density and in cuticle and velamen thickness among the *O. maculata* populations at the four localities studied. Besides the relationship with light intensity mentioned before, stomatal density is closely related to other environmental factors (Eames & MacDaniels, 1953; Esau, 1960; Wilkinson, 1979), such as light quality (Schoch *et al.*, 1984; Liu-Gitz, Britz & Wergin, 2000), humidity (Serna & Fenoll, 1997), UVB radiation (Dai *et al.*, 1995), drought signals (Quarrie & Jones, 1977; Franks & Farquhar, 2001), ozone (Pääkkönen, Holopainen & Kärenlampi, 1997) and atmospheric carbon dioxide concentration (Woodward, 1987; Holroyd, Hetherington & Gray, 2002). A greater number of stomata could increase the efficiency of gaseous exchange when relative humidity is high and, therefore, the danger of dehydration is minimal (Lleras, 1977; Fahn, 1990; Valladares & Niinemets, 2008). Cuticle thickness is an attribute that responds dynamically to internal and external stimuli (Wilkinson, 1979; Müller & Riederer, 2005; Bargel *et al.*, 2006). Variations in light levels, temperature, relative humidity, rain-induced erosion and wind are factors which depend upon the season, vary among places and affect the deposition of wax on the surface of the cuticle, and, therefore, the thickness of the cuticle (Esau, 1960; Withner *et al.*, 1974; Baker & Hunt, 1986; Hadley & Smith, 1989; Fahn, 1990; Sinclair, 1990; Gülz & Müller, 1992; Faini, Labbe & Coll, 1999; Dodd & Afzal-Rafii, 2000; Jenks *et al.*, 2002; Percy *et al.*, 2002; Dodd & Poveda, 2003). On the other hand, it has been shown that the rate of maturation and size of the velamen cells tend to increase with rise in relative humidity (Dycus & Knudson, 1957; Oliveira & Sajo, 1999b) and consequently influence its thickness. Taking into account the examples above, the differences found in our study could be result of the relationships between anatomical traits and environmental and microhabitat variables that were not measured, such as relative humidity, light percent at soil level, temperature at soil level, wind flow and litter thickness. On the other hand, the general macroclimatic conditions of the four localities shows that *O. maculata* in this region of Mexico is growing under humidity and middle to high precipitation; contrasting with Bone *et al.* (2015) findings, who pointed out that *O. maculata* belongs to an Afro-Madagascan CAM lineage mainly restricted to dry/hot environments.

Following the defence function proposed in this study for the sunken glandular hairs, the differences

found between its densities among localities could be related to the intensity of threats caused by pests and diseases in each site. However, this hypothesis requires corroboration.

The combination and convergence of the anatomical characteristics of different organs of *O. maculata* constitute adaptations which coincide with terrestrial and the epiphytic habits and xerophytic, high light intensity environments and humid, shaded environments. Furthermore, many of these characteristics offer protection in stressful environmental conditions and protection against herbivory. We could not relate the anatomical variations found in samples taken from the four localities with their general macroclimatic conditions, but these variations might indicate that, once established, *O. maculata* is then capable of responding to specific microenvironmental stimuli.

The complex of anatomical characteristics described in this study could be a contributory factor to the successful expansion of *O. maculata*. However, although it has been suggested that this species is probably displacing the native terrestrial orchids *Pelexia hondurensis* Ames in Bonampak (Hágsater *et al.*, 2015) and *Wullschlaegelia calcarata* Benth and *Prescottia stachyodes* (Sw.) Lindl. in Puerto Rico (Cohen & Ackerman, 2009), there have been no studies demonstrating its impact on the communities where it becomes established. For that reason, supporting studies should be carried out to cover other aspects, such as the characterization of the microenvironments where *O. maculata* thrives, demography, reproductive strategies and mycorrhizal associations to explain fully the invasive behaviour of this African terrestrial orchid and, if necessary, design efficient control strategies.

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