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Ethylene Modulates the Developmental Plasticity and the Growth Balance Between Shoot and Root Systems in the In Vitro Grown Epiphytic Orchid *Catasetum fimbriatum*

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Abstract The epiphytic habitat is potentially one of the most stressful environments for plants, making the effective developmental control in response to external cues critical for epiphyte survival. Because ethylene mediates several abiotic stresses in plants, here, we have examined the ethylene influence in both shoot and root systems of the epiphytic orchid *Catasetum fimbriatum*. Under controlled conditions, ethylene production was quantified during an entire growth cycle of *C. fimbriatum* development in vitro, while treatments modulating either ethylene concentration or perception were carried out over the early growth phase of these plants. After treatments, growth measurements and histological features were studied in both shoot and root tissues. Ethylene production showed a decreasing trend over the period of organ elongation; however, it increased considerably when leaves were shed, and a new axillary bud was initiating. The early exposure of young plants to higher concentrations of ethylene triggered morphogenic responses that included root hair formation instead of velamen, and a combination of inhibitory effects (decreases in both stem enlargement and cellular/organ elongation) and inductive effects (increases in leaf and root formation, bud initiation and cellular thickening) on plant growth, which favored biomass allocation to roots. Conversely, inhibition of ethylene perception over the plant growth phase generally resulted in the opposite morphogenic responses. Our data indicate that periodic variations in ethylene concentration and/or sensitivity seem to modulate several developmental features in shoot and root systems of *C. fimbriatum* which could have adaptive significance during the growing phase of this epiphytic orchid.

Keywords *Catasetum fimbriatum* · Developmental plasticity · Epiphytic orchid · Ethylene · Velamen

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Introduction

Ethylene is a gaseous phytohormone that modulates a wide variety of plant adaptive responses and developmental steps ranging from germination to senescence. Several ethylene-regulated responses are essential for tolerance, fitness, and survival of plants under environmental stresses. In fact, ethylene production is generally stimulated under adverse conditions, making this hormone a recognized signal of potential stress in plants. Thus, acting as a key regulatory and signaling molecule, ethylene contributes to adjust the plant's phenotype and physiology to the dynamic conditions of its surroundings (Pierik and others 2007; Lin and others 2009; Potters and others 2009; Yoo and others 2009; Vandenbussche and others 2012).

The epiphytic niche is virtually one of the most stressful for plants because epiphytes suffer frequent resource shortage and mechanical disturbance (Withner 1959; Benzing 1990; Sinclair 1990; Moffett 2000). In spite of the numerous abiotic stresses endured over their lifetime, epiphytes represent approximately 10 % of all vascular

plant species, and they can be found in many ecosystems. Moreover, epiphytism is importantly represented by Orchidaceae, one of the largest and most diverse families of flowering plants, with about 72 % of the orchid species living as epiphytes. The adaptive success of epiphytes is closely related to a variety of morphological adaptations and highly plastic physiological mechanisms for enhancing the uptake of resources available in the environment, and using them efficiently (Kress 1986; Pridgeon 1986; Gentry and Dodson 1987; Goh and Kluge 1989; Lüttge 1989, 2010; Benzing 1989, 1990; Zotz and Hietz 2001; Gravendeel and others 2004; Silvera and others 2005, 2009). The most striking characteristic of several epiphytic orchids is the development of enlarged, resource-storing stems called pseudobulbs, and an extensive aerial root system (Benzing 1990; Sinclair 1990). The roots of epiphytic orchids are considered the organs responsible for trapping and absorbing the bulk of water and mineral solutes, with the velamen and the exodermis representing key specializations for these purposes (Benzing and others 1982; Pridgeon 1986, 1987).

Catasetum is a neotropical genus of orchids with species that are usually deciduous epiphytes which follow the well-established seasonal pattern of growth and rest over the development. During the active growth phase, these plants benefit from abundant moisture and nutrients for a fast and robust development of new organs. Besides, much of the root system of *Catasetum* species can be embedded in rotting wood or organic debris nearly cut off from light and air—a habitat which defines them as humus epiphytes (Benzing 1990; Lacerda 1995; Zotz and Winter 1994). Although it is well accepted that *Catasetum* orchids can show notable morphological flexibility during their seasonal development (Gregg 1982; Benzing 1990; Zimmerman 1990, 1991), studies based on specific signals controlling the morphogenesis of these plants are still hampered by experimental difficulties. However, some insightful studies with *Catasetum* species under controlled in vitro conditions have revealed an astonishing plasticity in physiological and morphological responses triggered by specific environmental and endogenous cues. For example, Suzuki and others (2004, 2010) have demonstrated an important role of light in the establishment of a source-sink competition involving the shoot apex activity and the pseudobulb enlargement in *Catasetum fimbriatum*. In addition, it was shown that root tips isolated from *Catasetum* species are able to directly convert into buds, which in turn, give rise to new plants (Kerbauy 1984; Kraus and Monteiro 1989). Interestingly, previous evidence has also indicated that these events of morphogenesis described for *Catasetum* are affected by ethylene to some extent (Kerbauy and Colli 1997; Peres and others 1999; Suzuki and Kerbauy 2006). The above mentioned results suggest that modulation of ethylene concentration and/or signaling could be important for regulating some architectural traits in both shoot and root systems of *Catasetum* species; however, the influence of this hormone on the developmental plasticity of these plants is still unclear.

Moreover, abiotic stresses affecting epiphytes often co-occur and can promote similar morphological characteristics, and thus, sorting out the effects of either one is particularly challenging (Benzing 1990; Sinclair 1990). However, it is currently suggested that a range of distinct environmental constraints can trigger similar stress-induced morphogenic responses in several nonorchid plants, with ethylene participating as a key element in signaling and response mediation under stressful conditions (Pierik and others 2006, 2007; Potters and others 2007, 2009). This set of information prompted us to initially explore a hypothetical link between this hormone and the morphogenic responses that could have adaptive significance during the growing phase of *C. fimbriatum* plants subjected to unfavorable environmental conditions. Therefore, the present study used the controlled experimental conditions offered by in vitro plant culture to assess the hypothesis of whether the growth pattern observed during *C. fimbriatum* development under controlled conditions might be connected with variation in ethylene production and/or sensitivity. We specifically inquired whether modulating either ethylene concentration or its perception over the early growth phase of newly formed organs would interfere in the subsequent developmental patterning of both shoot and root systems. Concurrently, histological responses triggered by the ethylene-modulating treatments were also studied in shoot and root tissues of this epiphytic orchid.

Materials and Methods

Plant Material and Growth Conditions

Plants of *Catasetum fimbriatum* Morren Lindl (Orchidaceae) were obtained by micropropagation using a previously established pool of genetically identical plants that constitute the CFC1 genotype (Peres and Kerbauy 1999). *C. fimbriatum* micropropagation was based on the procedure described by Suzuki and others (2004), which uses etiolated nodal segments as explants for clone propagation of this species. Based on this method, light-grown plants bearing fully developed pseudobulbs had all leaves and older roots removed and were, subsequently, transferred to etiolating medium consisting of Vacin and Went (1949) macronutrients, micronutrients of Murashige and Skoog (1962), thiamine 0.01 %, soy peptone 0.1 %, sucrose 2 %, Phytagel® 0.2 %, and pH 5.8. Fifteen plants were

inoculated in each 1-l flask with 100 ml of this medium and then maintained for 6 months in the dark at 25 ± 2 °C to induce shoot etiolation. For propagation and growth of the experimental plants, nodal explants isolated from the etiolated material were inoculated in a growth medium prepared with Vacin and Went (1949) macronutrients, Murashige and Skoog (1962) micronutrients, activated charcoal 0.1 %, sucrose 2 %, agar 0.7 %, and pH 5.8. Fifteen explants were aseptically inoculated in each 250-ml Erlenmeyer flask with 80 ml of growth medium. The plant material was cultured on a semisolid medium with agar for the formation of a support matrix which kept the plants from being submerged in the medium. Besides, the culture medium was adapted to meet the closest conditions that were possible for *Catasetum* growing substrate, such as the addition of activated charcoal. Activated charcoal has a very intricate network of pores, a large surface area, and volume that gives it a unique adsorption capacity of inhibitory compounds from the medium (Baker and others 1992). This type of medium composition has been reported as efficient for inducing root development in micropropagated shoots of several orchid species. It is also suggested that the activated charcoal could benefit increased rooting in such orchids by creating a partially darkened environment in the medium which simulates the substrate conditions where these plants naturally inhabit (Yan and others 2006; Thomas 2008). Therefore, all explants were incubated under 25 ± 2 °C and 16 h of light (55 μmol m^{-2} s^{-1}) for nodal bud release and subsequent plant development.

Determination of Ethylene Production

To determine the rate of ethylene production by *C. fimbriatum* plants at different phases of development, the production of this hormone was measured in 1-, 2-, 4- and 8-month-old plants grown in vitro, which corresponded, respectively, to the following developmental phases: initial shoot and root organogenesis (Fig. 1a, e), fast organ formation and elongation (Fig. 1b, f), pseudobulb enlargement (Fig. 1c, g), and leaf shedding (Fig. 1d, h). To guarantee equivalent experimental conditions at the moment of ethylene quantification, all plants were transferred to 125-ml Erlenmeyer flasks filled with 30 ml of fresh growth medium (30 individuals per flask; three flasks per plant age), and maintained for 15 days under 25 ± 2 °C and 16 h of light at 55 μmol m^{-2} s^{-1}. Following this period of acclimatization, the flasks were closed with rubber stoppers, then the internal gaseous composition was flushed for 5 min with a continuous flux (3 l min^{-1}) of ethylene-free air, and these air-tight flasks were incubated for 48 h at the above described growth conditions. After this period, samples of 1–10 ml were collected from the headspace of each flask using a gas-tight syringe and then injected into a gas chromatograph (HP-6890) in pulsed split less mode with a flame-ionization detector at 250 °C and a column HP-Plot Q (30 m, I.D. 0.53 mm). Helium was the carrier gas used in a flow rate of 1 ml min^{-1} with a column isothermal at 30 °C. Ethylene quantification was performed according to the peak area of C_2H_4 standards used for calibration curves.

Treatments and Experimental Conditions

To test whether variation in ethylene concentration or perception would affect the developmental pattern of *C. fimbriatum* roots and shoots, an experimental approach using treatments with different concentrations of either ethylene or the inhibitor of ethylene perception, 1-methylcyclopropene (1-MCP), was employed. Single 1-month-old plants with one initiated unit of root and shoot (each one nearly 15 ± 5 mm long) were transferred to air-tight assay tubes (250 mm length × 25 mm diameter) filled with 50 ml of the growth medium previously described and sealed with rubber stoppers. Ethylene gas was injected through the rubber stopper to a final concentration of 5, 50, or 500 μl l^{-1}, whereas proper volumes of the gaseous 1-MCP were added in the headspace of the assay tubes to a final concentration of 0.5, 5, or 50 μl l^{-1}. The preparation of 1-MCP followed the manufacturer's instructions (SmartFresh® powder 0.14 %).

During the subsequent five weeks of plant development (a period that corresponds to the higher rate of organ formation and elongation, Fig. 1b, f), all treatments were renewed weekly by flushing the headspace of each tube with a 3 l min^{-1}continuous flux of ethylene-free air for 1 min, followed by the reestablishment of the final concentrations of ethylene or 1-MCP specific for each treatment. The control was conducted with plants cultivated under the same conditions as described for ethylene and 1-MCP treatments, except that control tubes had their headspace content renewed weekly only with ethylene-free air. Each experimental condition was performed with 30 plants incubated in independent assay tubes in a growth chamber with 16 h of light (55 μmol m^{-2} s^{-1}) and temperature at 25 ± 2 °C. After the 5-week treatments, all plants were maintained under the same growth conditions until they were 4 months old to measure the impact of an initial period of ethylene modulation on the following seasonal pattern of new organ formation and their subsequent development.

Growth and Morphological Measurements

All the growth measurements were performed when plants were 4 months old (the period when organ elongation

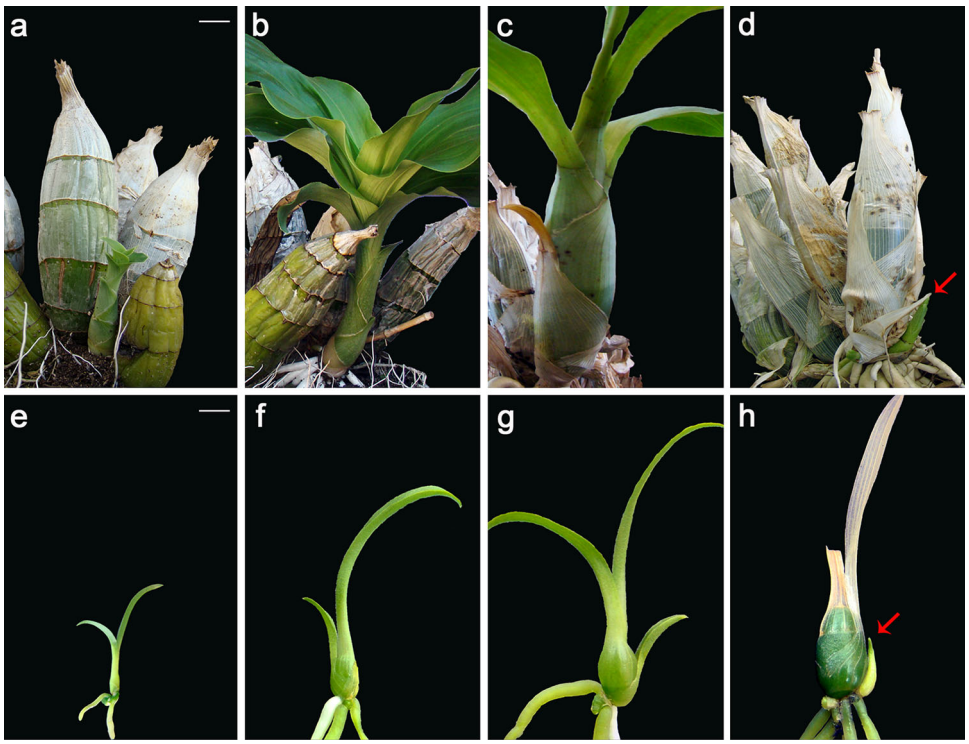

Fig. 1 Comparison between the seasonal regime of growth and rest in *Catasetum fimbriatum* plants grown ex vitro (**a–d**) and in vitro (**e–h**). **a**, **e** Initial shoot and root organogenesis in approximately 1-month-old plants; **b**, **f** Fast organ formation and elongation (stem, leaves and roots) in approximately 2-month-old plants; **c**, **g** Pseudobulb enlargement in approximately 4-month-old plants; **d**, **h** Leaf shedding in approximately 8-month-old plants with *arrows* indicating the new axillary buds that will generate the next seasonal plants; *scale bars*: **a–d** 10 mm; **e–h** 5 mm

decreases and pseudobulb enlargement happens, Fig. 1c, g). Hence, all 4-month-old plants from each treatment were harvested, the roots were thoroughly washed in distilled water, and the plants were separated into shoot and root systems. These samples were weighed to determine fresh weight (FW) and, subsequently, dried at 60 °C to determine the dry weigh (DW), with the difference between both these data used to determine the water content. The root (or shoot) mass ratio was calculated as root (or shoot) weight divided by the total (root plus shoot) weight (for FW, DW, and water content data). The measurements of root and shoot elongations were performed by measuring the length of the both organs before and after the treatments, and the difference between the two sets of data was used to compute the organ elongation, considering only the older root and shoot of each plant. Leaf blades were excised from stems, leaf area was determined (total area of all leaves per plant), and the numbers of leaves, shoots, and roots per plant were also recorded.

Histological Analyses

Intact leaves, shoot apices, and 1-cm-long root fragments (isolated from the middle portion of the oldest root) of control, ethylene- and 1-MCP-treated plants were used for histological analyses. The samples were fixed for 24 h at room temperature in Karnovsky's solution (1965) modified by the addition of glutaraldehyde/paraformaldehyde (4:1, v/v) in 0.1 M phosphate buffer (pH 7.2). The fixed material was dehydrated in a graded ethanol series until a concentration of 70 % was reached and, subsequently, submitted to hand-cut sections. The shoot apices were sliced in longitudinal sections, whereas, leaves and root fragments were sliced in both transverse and longitudinal sections. At least three samples collected from different plants of each treatment were processed, and all the obtained cuttings from each sample were mounted on glass slides and stained with 0.05 % toluidine blue O (CI 52040) in 0.1 M phosphate buffer, pH 6.8. Photomicrographs were taken with a digital Leica® DFC320 camera on a Leica® DM LB microscope (Wetzlar, Germany).

Statistical Analyses

The mean ± standard error values of the results were analyzed by one-way ANOVA, and the significant differences among the treatments were compared by the Tukey's test at 5 % probability using the JMP software (SAS System for Windows, version 5.0.1a).

Results

As illustrated in Fig. 1, there is a considerable similarity between the rhythmic pattern of development in *C. fimbriatum* plants under both natural (ex vitro) and controlled (in vitro) conditions. The maintenance of such a seasonal growth rhythm during in vitro development was followed

Table 1 Ethylene production by *Catasetum fimbriatum* plants at different phases of development in vitro

Plant age	Developmental phase	Ethylene production ($\mu l\ g^{-1}$ FW h^{-1})
1-Month old	Initial shoot and root organogenesis	66.11 ± 10.68b
2-Month old	Fast organ formation and elongation	50.56 ± 2.65bc
4-Month old	Pseudobulb enlargement	21.92 ± 6.67c
8-Month old	Leaf shedding and new bud initiation	198.64 ± 5.17a

Ethylene levels are mean values of three replicates ± standard error. Each replicate contained thirty plants. Different letters following each value indicate statistically significant differences

Categories of plant age/developmental phase used to generate the table data that correspond to those in Fig. 1

by the changes in ethylene evolution (Table 1). The ethylene production by these plants depended on their developmental phase with a decreasing trend during the first 4 months of plant development; the period when new organs (stem, leaves and roots) were rapidly forming and elongating (Table 1; Fig. 1e–g). However, after 8 months of plant development, the ethylene production was considerably higher, coinciding with the termination of a growth cycle, when leaves are shed, the pseudobulb enters into the dormancy state, and a new axillary bud is formed at the base of this organ (Table 1; Fig. 1h).

Treatments with either ethylene or 1-MCP during the initial growth phase of *C. fimbriatum* plants showed clear modifications in both root and shoot morphologies (Fig. 2). The chronic exposure of young plants to higher levels of ethylene during the 5 weeks of treatment induced the classical response of severe growth impairment (Fig. 2a), which was partially alleviated when plants were allowed to grow without ethylene treatment renewals for two extra months (Fig. 2b). Conversely, the supply of 1-MCP during the 5 weeks of treatment was sufficient to trigger opposite morphogenic effects compared with those induced by exogenous ethylene over the same period (Fig. 2a), and the following 2 months of plant growth in the absence of treatment renewal amplified the 1-MCP effects (Fig. 2b). Compared to controls, all treatments using either ethylene or 1-MCP caused an increase in the total fresh weigh (FW), dry weigh (DW), and water content in *C. fimbriatum* plants, with the highest amounts for all these parameters found in the ethylene-treated plants (Fig. 3). In general, as the ethylene concentration was elevated, there was an increase in all analyzed parameters for root ratios (FW, DW and water content) with the opposite trend for all shoot ratios. In contrast, 1-MCP treatments resulted in a tendency to elevate shoot ratios and decrease root ratios for these same parameters (Fig. 4).

As shown in Fig. 5a, ethylene treatments significantly reduced shoot elongation in all tested concentrations, whereas, this same parameter tended to increase in 1-MCP-treated plants. In addition, 1-MCP caused a notable stimulation in leaf development of *C. fimbriatum* plants, especially by inducing leaf blade expansion, thus, increasing the total leaf area (Figs. 5b, 6c). On the other hand, ethylene treatments resulted in premature shoot branching (Figs. 5a, 6b) and severely impaired pseudobulb and leaf development, hence, resulting in plants with numerous, though smaller, leaves and shoots (Figs. 5, 6b). When compared to controls and 1-MCP-treated plants, the leaf-forming rate at the shoot apex of the ethylene-treated plants was increased greatly due to long-term maintenance of the shoot apical meristem (SAM) activity (Fig. 6d–e). Leaf growth inhibition in response to high levels of ethylene occurred concomitantly with reduced elongation and increased thickening of cells in mesophyll, epidermis, and vascular tissues (Fig. 6f–p). Ethylene treatments promoted strengthening of fiber bundles crossing the mesophyll with prominent development of stegmata associated with the surface of supporting tissues (Fig. 6i–n). Moreover, raphide idioblasts in the mesophyll (Fig. 6j) and sunken leaf hairs on the adaxial leaf surface were observed more frequently in ethylene-treated plants, especially in younger leaves enclosing the SAM (Fig. 6q–s).

Figure 7 shows that ethylene induced the development of new adventitious roots in 4-month-old plants of *C. fimbriatum*; however, high levels of this hormone severely inhibited the elongation of older roots (those already formed during the treatment period). Conversely, 1-MCP tended to inhibit the formation of new adventitious roots but did not considerably affect relative root elongation (Fig. 7). In addition, modulation of either ethylene levels or perception caused several alterations in cellular configuration of *C. fimbriatum* roots (Fig. 8). Essentially, ethylene treatments induced a specific pattern of cellular differentiation in most root tissues, which included intense and ectopic root hair formation (instead of velamen) (Fig. 8a, b, d, e), decreased longitudinal elongation and transverse widening of cortical cells (Fig. 8d, e), reduced diameter of both vascular cylinder (Fig. 8g, h) and exodermal cells (Fig. 8j, k), and increased cell wall thickenings in the cortex (Fig. 8m, n). In general, 1-MCP treatments caused opposite cellular effects to those described for ethylene (Fig. 8c, f, i, l, o); however, the inhibition of ethylene perception by 1-MCP treatment displayed the differentiation of fewer and less thickened velamen layers when compared to the control (Fig. 8a, c, d, f). Furthermore, ethylene treatments induced the ectopic formation of buds in the root tips of *C. fimbriatum* plants, and this

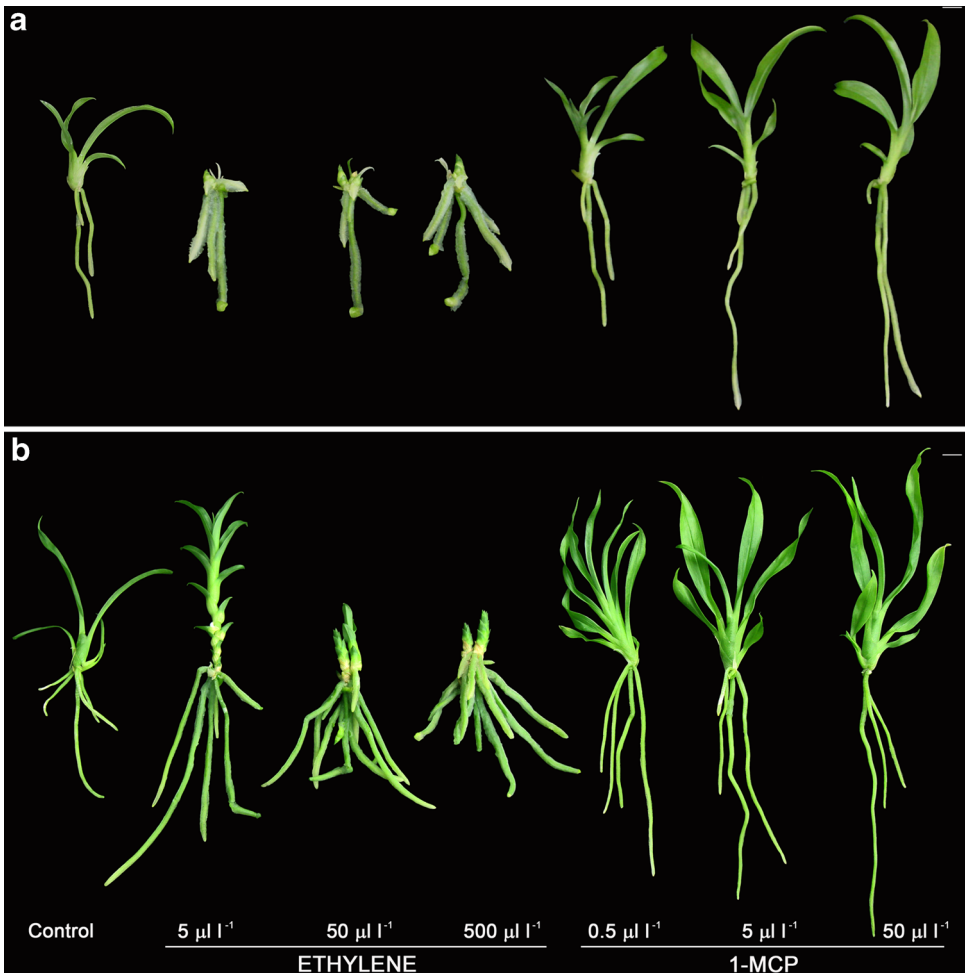

Fig. 2 Morphological responses of *Catasetum fimbriatum* plants to different concentrations of either ethylene or 1-MCP (an inhibitor of ethylene perception) imposed during the initial phase of plant development. **a** Morphology of 2-month-old plants at the end of treatments, illustrating the rapid effects of ethylene and 1-MCP. **b** Morphology of 4-month-old plants maintained in culture for two additional months after the end of ethylene or 1-MCP treatments, illustrating the long-term effects of these substances; *scale bars* 10 mm

morphogenic process occurred more frequently in older roots (Fig. 9). The general effects of ethylene on the *C. fimbriatum* developmental patterns described in this study (bud formation in the root tips, intense root hair differentiation, numerous small leaves formed in the shoot system, and the early shoot branching) were also observed during the development of these newlyformed ectopic buds from root tips maintained under ethylene treatment (Fig. 9).

Discussion

Taking advantage of the observation that *C. fimbriatum* plants maintain the rhythm of growth and rest under in vitro conditions (Fig. 1), we found that different developmental phases of newly formed plants correlated with specific levels of ethylene production (Table 1). As a general view, increased ethylene concentrations (produced by plant tissues or exogenously supplied) seemed to play an inductive role in initiating new organs during the early developmental phase of *C. fimbriatum* plants (Table 1; Figs. 2, 5, 7). However, the growth release of newly formed shoots and roots was only allowed when ethylene concentration and/or perception was diminished during organ expansion in both shoot and root systems of this orchid (Table 1; Fig. 2). These observations suggest that a temporary fluctuation in ethylene production is likely to be an important signal modulating not only the initiation of new organ primordia in *C. fimbriatum* plants but also the growth release of the newly formed organs. Whether this evidence reflects the ethylene production and its morphogenic effects during the development of *C. fimbriatum* plants in their natural habitat is uncertain and deserves further investigation. However, the coordination between ethylene evolution and the developmental rhythm of *C. fimbriatum* plants growing in vitro are likely to be connected with depletion of the substratum resources because a temporal correlation was detected between these parameters presented here, and the concomitant decrease in pH, water content, and ammonium levels in the culture medium where *C. fimbriatum* plants were cultivated (data not shown). Hence, this evidence highlights the possible role of ethylene as a signaling molecule during environmental constraints imposed by depletion of substrate

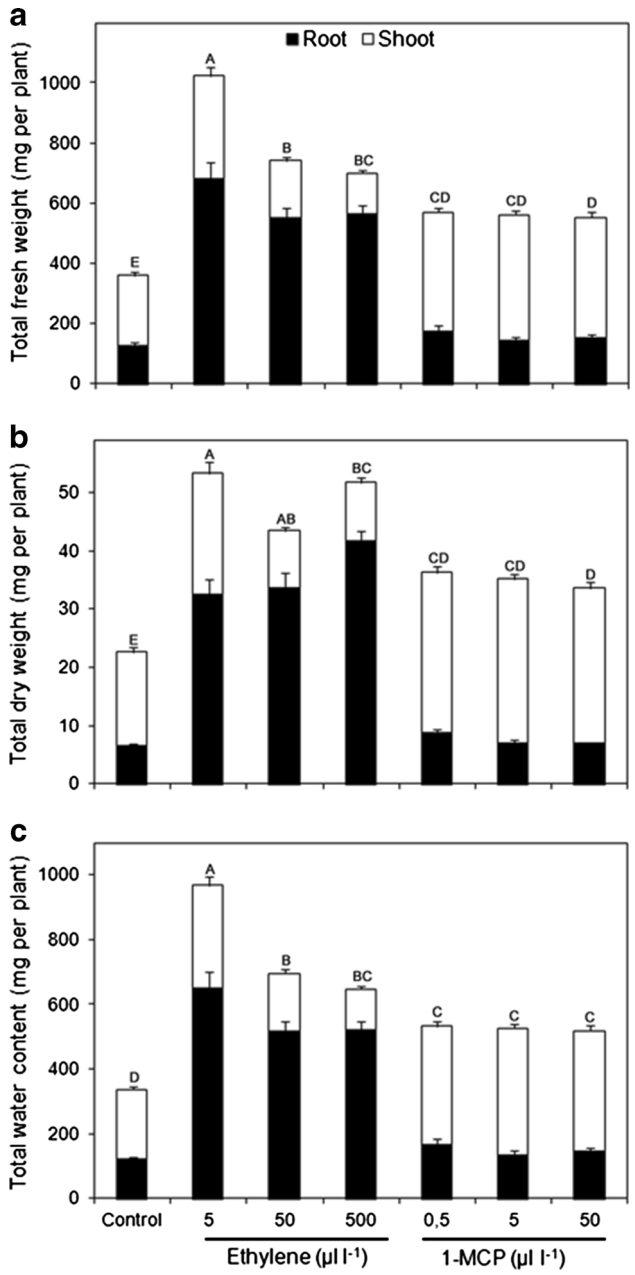

Fig. 3 Effects of ethylene and 1-MCP treatments on the total fresh weight (**a**), dry weight (**b**), and water content (**c**) in the shoot and root systems of 4-month-old *Catasetum fimbriatum* plants. *Columns* show the sum of results for shoot (*white boxes*) and root (*black boxes*) systems. Data are mean values of 30 replicates ± standard error. *Different letters* indicate statistically significant difference among the total (shoot + root) values

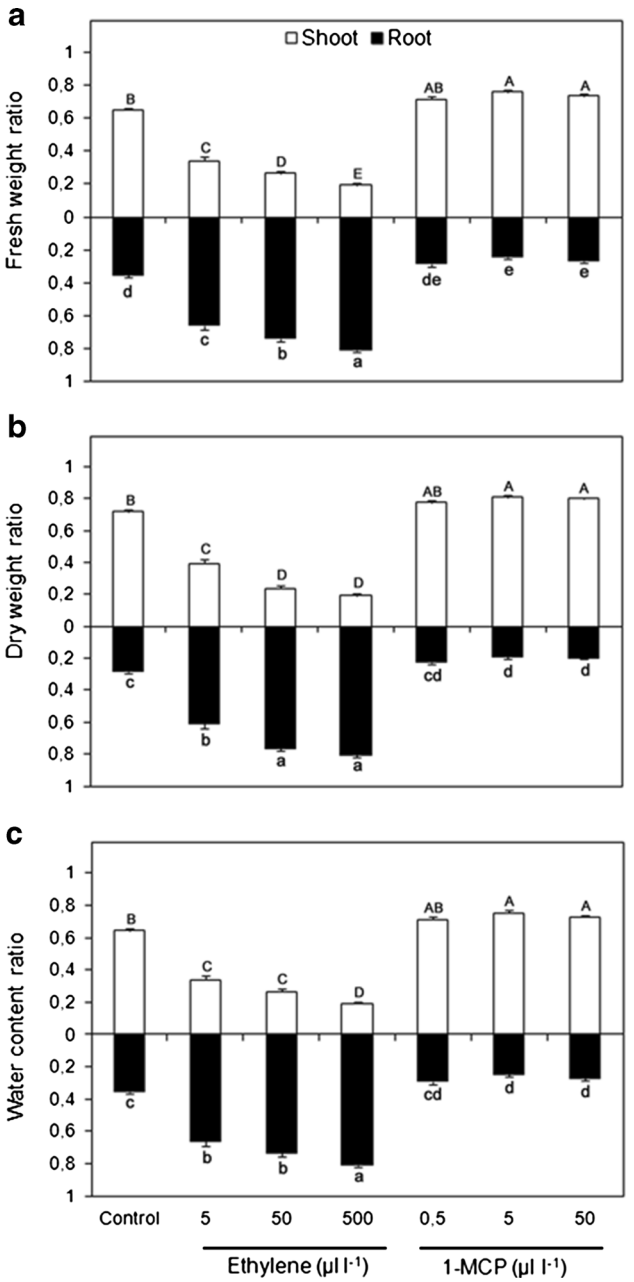

Fig. 4 Effects of ethylene and 1-MCP treatments on shoot (and root) fresh weight ratio (**a**), dry weight ratio (**b**), and water content ratio (**c**) of 4-month-old *Catasetum fimbriatum* plants. Shoot (or root) ratio = shoot (or root)/total per plant. *Columns* show results for shoot (*white boxes*) and root (*black boxes*) systems. Data are mean values of 30 replicates ± standard error. *Different letters* indicate statistically significant differences: *capital* and *lowercase letters* refer to shoot and root data, respectively

resources where *C. fimbriatum* plants were grown; though, the participation of other environmental cues controlling the development of this orchid through ethylene mediation in its natural habitat cannot be rule out. Besides, the results obtained with 1-MCP application during the early developmental phase of *C. fimbriatum* in vitro indicated that controlling ethylene action by reducing its perception, and/or concentration in the headspace of flasks where these orchids are cultivated is decisive for the success of plant micropropagation and subsequent orchid development in vitro.

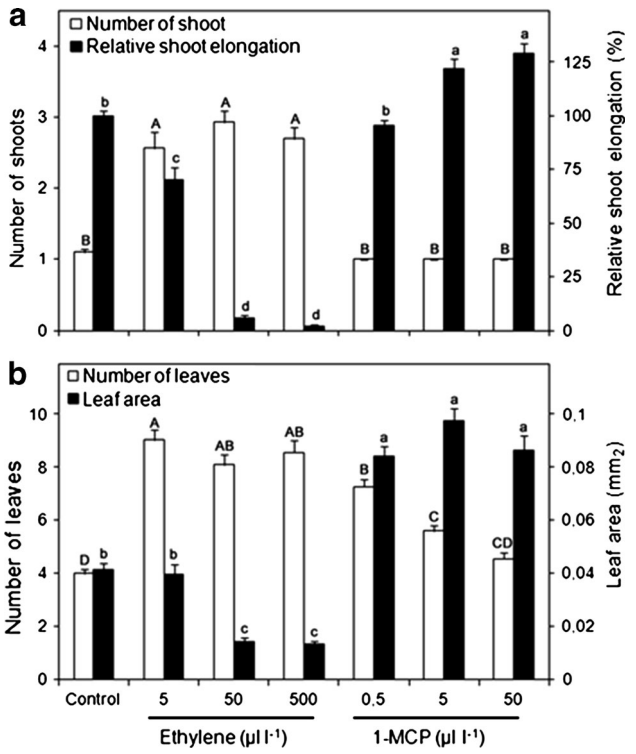

Fig. 5 Effects of ethylene and 1-MCP treatments on shoot development of 4-month-old *Catasetum fimbriatum* plants. All data are mean values of 30 replicates ± standard error: **a** Number of shoot (*white columns*) and relative shoot elongation (*black columns*). Number of shoots was recorded per plant, and the relative shoot elongation was calculated as the difference between the older shoot length after and before the treatments, and the difference between the two sets of data was used to compute the organ elongation relative to the control (control value was arbitrarily considered as 100 %). *Different letters* above columns indicate statistically significant differences: *capital* and *lowercase letters* refer to number of shoot and relative shoot elongation data, respectively; **b** Number of leaves (*white columns*) and leaf area (*black columns*). Both data sets correspond to records per plant. *Different letters* above columns indicate statistically significant differences: *capital* and *lowercase letters* refer to number of leaves and leaf area data, respectively

Alterations in ethylene concentration and/or perception over the early developmental phase of *C. fimbriatum* also modulated several histological aspects in both shoots and roots of this orchid denoted by a high plasticity in tissue specialization that might be related to cellular protection and resistance to harsh conditions during the initial organogenesis of new plant structures. For example, ethylene treatments induced the development of heavier conical structures identified as stegmata on the surface of most vascular and fiber bundles traversing the *C. fimbriatum* mesophyll (Fig. 6m) which could greatly strengthen the leaf blade of many epiphytic orchids under severe environments (Rasmussen 1986). Moreover, ethylene-treated plants formed numerous sunken leaf hairs mainly located on the adaxial surface of *C. fimbriatum* younger leaves (Fig. 6g, q–s). However, sunken leaf hairs have been recorded for all Catasetinae as occurring in low density on both leaf surfaces (Stern and Judd 2001). Thus, the pronounced formation of bulky leaf hairs on ethylene-treated leaves of *C. fimbriatum* indicates a potential protective role to younger tissues, especially those enclosing the shoot apex, against direct contact with stressful environments, such as low humidity in the surrounding atmosphere, as suggested by Sinclair (1990) for other epiphytic orchids. Another cellular feature with enhanced occurrence in ethylene-treated leaves was raphide idioblasts (Fig. 6j), which are specialized cells that accumulate many needle-shaped calcium oxalate crystals in their vacuoles (Franceschi and Nakata 2005). In spite of the potential problem of accumulating calcium as raphide crystals in developing tissues, this strategy might benefit *C. fimbriatum* development after suspending plant exposure to high levels of ethylene (that is, period of harsher abiotic conditions) because a great deal of evidence indicates that raphides disappear during tissue maturation, and where new growth is very active and the availability of calcium is limited (Franceschi and Nakata 2005).

Moreover, ethylene-treated leaves of *C. fimbriatum* were very small, narrow, V-shaped (Fig. 6b), and showed both reduced elongation and increased thickening of cells in mesophyll, epidermis and vascular tissues (Fig. 6g, p). Among epiphytic orchids, small and narrow leaves are considered better adapted to exposed sites than larger ones because they lose heat more efficiently by convection and, therefore, do not heat as readily in full sun (Sinclair 1990). Besides, mature *Catasetum* foliage tends to be thin with little water-storage capacity and shows weak resistance to damage; thus, emphasizing the fact that these leaves are able to fully develop only under favorable environments (Benzing 1990; Sinclair 1990). Accordingly, young *C. fimbriatum* plants treated with ethylene invested massively in root system development (Figs. 3, 4, 7), as the aerial roots represent the organ responsible for epiphytic orchid anchorage, absorption of water and nutrients (Pridgeon 1986, 1987; Benzing 1990), and in the case of *C. fimbriatum*, the root tips formed ectopic buds with the potential to generate new cloned plants after the discontinuation of ethylene treatment (Fig. 9). As a general rule, a plant invests in an extensive root system when exposed to unfavorable conditions, resulting in a higher root to shoot ratio, which would benefit plants under certain unstable environments, as the faster root development can improve further exploitation and capture of resources available in the substrate (Garnett and others 2009). Hence, faster and more conspicuous root development was observed in *C. fimbriatum* plants after ethylene treatments with augmented adventitious roots formation (Fig. 7), increased cellular thickening and decreased diameter of both exodermis and

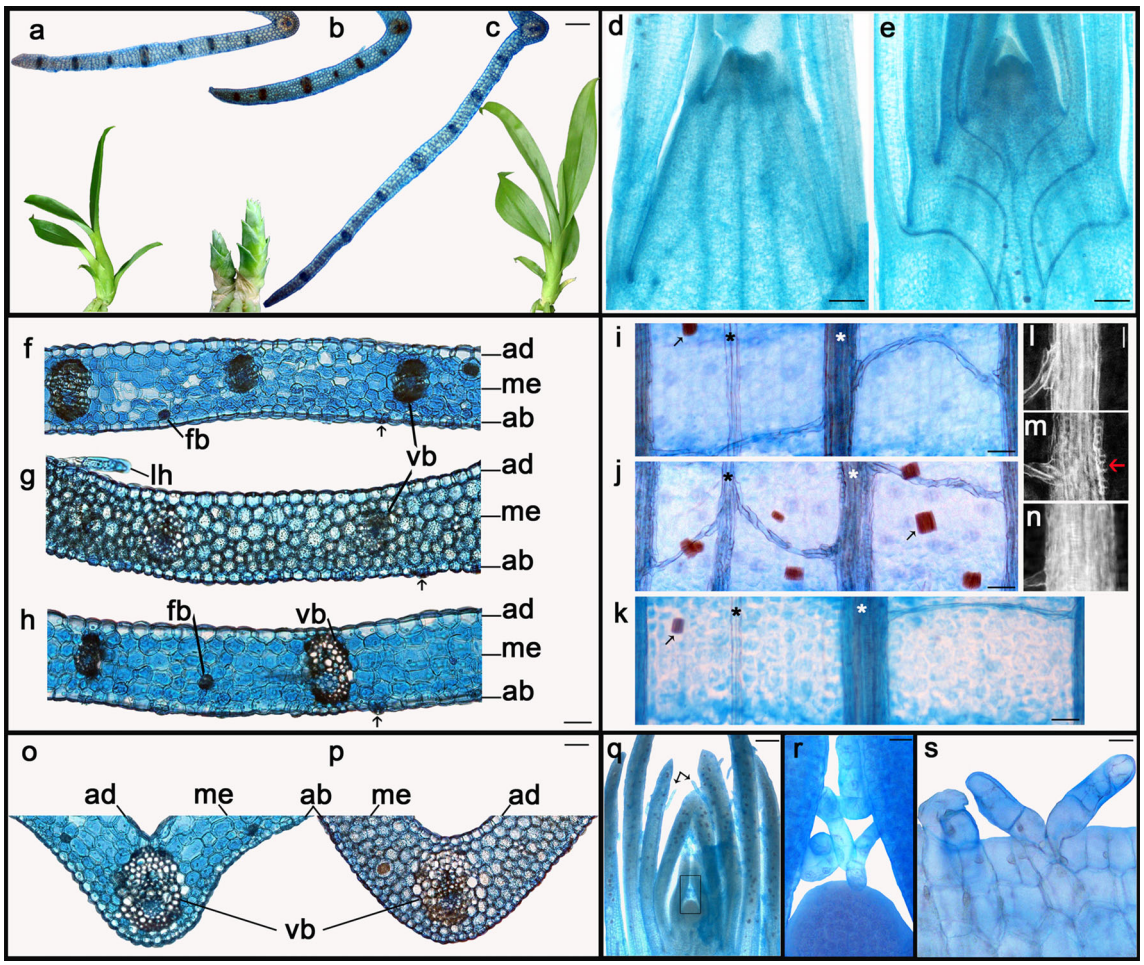

Fig. 6 Comparative view of histological effects induced by either ethylene or 1-MCP treatments on shoot development of 4-month-old *Catasetum fimbriatum* plants. **a–c** General view of transverse sections from the middle region of leaves submitted to control (**a**), ethylene (**b**) or 1-MCP (**c**) treatments; below each section is the corresponding plant used for histological preparations, *scale bar* 200 μm. **d, e** Shoot-apex longitudinal sections of control (**d**) and ethylene-treated (**e**) plants to comparatively show the shoot apical meristem (SAM) and the younger leaves formed (SAM morphology from 1-MCP-treated plants was similar to the control); *scale bars* 150 μm. **f–h** Transverse sections from leaf blade showing vascular bundles (*vb*) scattering in a homogenous mesophyll (*me*) and stomata (*arrows*) on the abaxial surface (*ab*); evident presence of fiber bundles (*fb*) in control (**f**) and 1-MCP-treated (**h**) leaves, and leaf hair (*lh*) on the adaxial surface (*ad*) of ethylene-treated leaf (**g**); *scale bar* 50 μm. **i–k** Longitudinal sections from leaf blades showing vascular bundles (*white asterisks*) and idioblasts with raphides (*black arrows*). Fiber bundles (*black asterisks*) with thinner aspect in control (**i**) and 1-MCP-treated (**k**) leaves in comparison with the ethylene-treated (**j**) material; *scale bars* 50 μm. **l–n** Detail of vascular bundle surfaces showing more conspicuous stegmata (*arrow*) in ethylene-treated (**m**) leaves in comparison with control (**l**) and 1-MCP-treated (**n**) plants; *scale bar* 25 μm. **o–p** Midvein morphology of 1-MCP-(**o**) and ethylene-treated (**p**) leaves (midvein morphology in control leaves was similar to the 1-MCP-treated plants); *scale bar* 50 μm. **q–s** Morphological aspects of leaf hair development in plants treated with ethylene: (**q**) general view of ethylene-treated shoot apex showing numerous and long leaf hairs (*arrows*); the *box* highlights the SAM location, *scale bar* 200 μm, (**r**) detail of ethylene-treated SAM protected by young leaves with several leaf hairs and (**s**) morphological detail of the sunken leaf hairs mainly formed on the adaxial surface of young ethylene-treated leaves; *scale bars* 200 μm. All histological analyses were carried out with at least three organs from different 4-month-old plants submitted to control, 50 μl l^{-1} of ethylene or 5 μl l^{-1} of 1-MCP

vascular cylinder cells (Fig. 8h, k), and also a higher incidence of localized cell wall thickenings in the cortex cells (Fig. 8n), altogether, indicating an increased strengthening of internal tissues that might improve the root resistance under adverse conditions. In agreement with this suggestion, Stern and Morris (1992) recognized the development of bands of thickened cell walls as a protective morphological feature in epiphytic orchids to avoid tissue collapse during severe drought.

The flexible developmental nature of epidermal cells of *C. fimbriatum* was revealed by treating young plants with either high levels of ethylene or the inhibitor of ethylene perception, triggering the expression of specific morphogenic responses in the root epidermis as follows: high

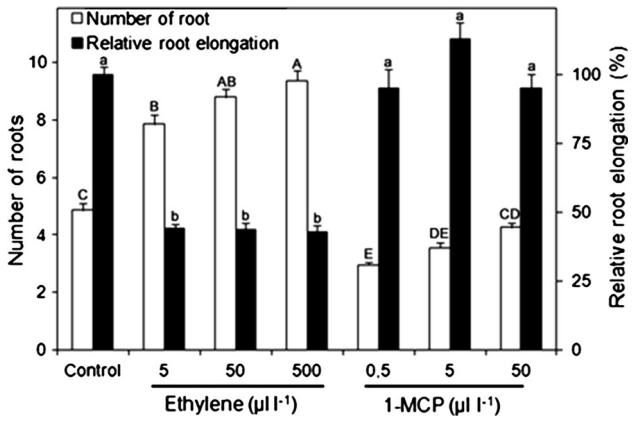

Fig. 7 Effects of ethylene and 1-MCP treatments on root development of 4-month-old *Catasetum fimbriatum* plants. Number of roots (*white columns*) and relative root elongation (*black columns*). Number of roots was recorded per plant, and the relative root elongation was calculated as the difference between the older root length after and before the treatments, and the difference between the two sets of data was used to compute the organ elongation relatively to the control (control value was arbitrarily considered as 100 %). All data are mean values of 30 replicates ± standard error. *Different letters* above columns indicate statistically significant differences: *capital* and *lowercase letters* refer to number of roots and relative root elongation data, respectively

levels of ethylene induced the differentiation of epidermal cells in abundant; long root hairs on the whole root surface (Figs. 8, 9), whereas, 1-MCP-treated roots showed the epidermal cells differentiated in a fewer layer velamen with less thickened cells in comparison with the control (Fig. 8c, f). The velamen has been described as a multiseriate epidermis that covers the root surface of epiphytic orchids (Benzing and others 1982; Pridgeon 1986, 1987; Benzing 1989, 1990); however, the physiological role of this unique structure is not yet fully understood (Goh and Kluge 1989). Regarding the potential functions suggested for orchid velamen, there are two current hypotheses that alternatively discuss the velamen as a structure that allows either absorption (Benzing and others 1982) or conservation (Dycus and Knudson 1957) of water and dissolved nutrients by orchid roots. Additional functions that are also ascribed to velamen include radiation reflection and mechanical protection of the internal root tissues, whereas, the protecting effects are usually associated with increased velamen thickness (Pridgeon 1987; Goh and Kluge 1989). On the other hand, the root hairs are consistently recognized as tubular projections from root epidermal cells that increase the root's surface area, playing an essential role in nutrient and water uptake, root exudation, root adhesion, and anchorage (Datta and others 2011). Therefore, the results obtained from ethylene modulating the developmental fate of epidermal cells in *C. fimbriatum* highlighted the suitability of such an experimental approach for further studies aiming to clarify the actual physiological roles of velamen versus root hair for epiphytic orchid roots. At this context, it is likely that ethylene effects in *C. fimbriatum* root epidermis might result from the crosstalk between this hormone and other signaling molecules because recent findings showed that higher levels of ethylene-modulated epidermal cell fate in *A. thaliana* roots by controlling auxin polar transport (Strader and others 2010).

Another intriguing morphogenic response modulated by ethylene in *C. fimbriatum* roots was the root tip conversion into new buds which agrees with previous results obtained with root tips isolated from *C. fimbriatum* that have shown the stimulatory effect of ethylene on accelerating the root tip conversion into buds (Kerbauy and Colli 1997; Peres and others 1999). However, until now, it has been challenging to discuss the possible adaptive significance of this morphogenic event for *C. fimbriatum* development because it was described as an organogenetic process triggered by root tip isolation from plants cultivated *in vitro*, whereas, attached roots were considered unable to convert into buds (Colli and Kerbauy 1993; Kerbauy and Colli 1997; Peres and Kerbauy 1999; Peres and others 1999). The results presented here revealed that ethylene is more than an accelerating signal for this morphogenic process because the treatment of young plants with this hormone was sufficient to trigger the conversion of root apices into buds, even when they were linked to intact plants (Fig. 9). Interestingly, other epiphytic orchids closely related to *C. fimbriatum* can also display bud development in isolated root tips, such as *Catasetum pileatum* (Kraus and Monteiro 1989), *Clowesia warscewiczii* (Kerbauy and Estelita 1996), and *Cyrtopodium punctatum* (Sánchez 1988). Accordingly, the root system of *Catasetum* plants in the natural habitat can display a relatively wide range of morphological flexibility when exposed to different nutritional and moisture conditions during the growth phase (Benzing 1990). Therefore, it will be interesting to verify whether forming root hair and ectopic buds via ethylene signaling would be a conservative developmental feature employed by other epiphytic orchids that, as *C. fimbriatum*, form a trash-basket impoundment where humus and debris can accumulate in their root system surroundings (Benzing 1990).

In summary, the results presented here suggest ethylene as an important signaling mediator to the flexible adjustments of *C. fimbriatum* development. The data obtained showed that fluctuation in ethylene concentration and/or perception can act as a powerful signal modulating not only the external morphology of this orchid but also the coordinated adjustment of histological characteristics. The ethylene-induced morphogenic responses observed in *C. fimbriatum* plants growing in vitro included the formation of an extensive root system with abundant root hairs, a high degree of cellular strengthening in both shoot and root

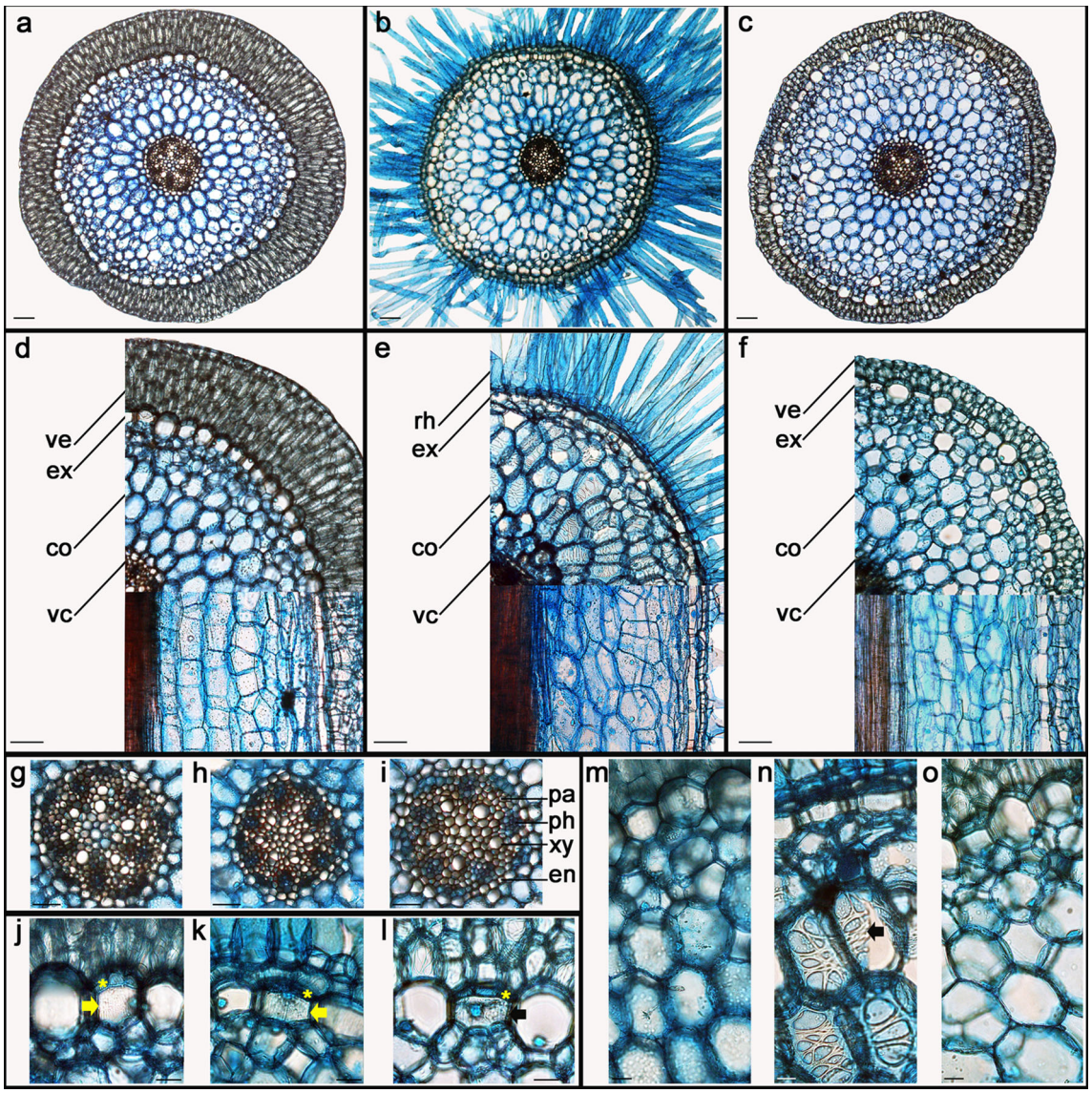

Fig. 8 Comparative view of histological effects induced by either ethylene or 1-MCP treatments on root development of *Catasetum fimbriatum* plants. **a–c** General view of transverse sections from the middle region of roots submitted to control (**a**), ethylene (**b**) or 1-MCP (**c**) treatments; *scale bars* 100 µm. **d–f** Comparative composition of both transverse and median-longitudinal root sections to show the following tissues listed from the outer to the inner type: epidermis differentiated into velamen (*ve*) or root hair (*rh*), exodermis (*ex*), cortex (*co*) and vascular cylinder (*vc*). Root epidermis from control (**d**) and 1-MCP treatment (**f**) formed mainly velamen (*ve*), while ethylene-treated epidermis formed root hair (*rh*) massively on all root surface (**e**); *scale bars* 100 µm. **g–i** Cross section of vascular cylinder showing the constituent tissues (*en*, endodermis; *ph*, phloem; *xy*, xylem; *pa*, parenchyma cells) from roots submitted to control (**g**), ethylene (**h**) or 1-MCP (**i**) treatments; *scale bars* 50 µm. **j–l** Detail of exodermis showing passage cell (*arrows*) with slightly thickened outer tangential wall (*asterisks*) in transverse sections from control (**j**), ethylene (**k**), and 1-MCP (**l**) treatments; *scale bars* 25 µm. **m–o** Detail of cortex cells showing the conspicuous presence of cell wall thickenings (*arrow*) in ethylene-treated root cortex (**n**) in comparison to cortex cells in control (**m**) and 1-MCP treatment (**o**); *scale bars* 25 µm. All histological analyses were carried out with at least three older roots from different 4-month-old plants submitted to control, 50 µl l^{-1} of ethylene or 5 µl l^{-1} of 1-MCP

supporting tissues, the initiation of several organ primordia maintained in the dormancy state, a short shoot system with shoot apical meristem still active and relatively well protected, and ectopic buds formed in root tips with the potential to generate new cloned plants. Altogether, these ethylene-induced morphogenic features seemed to enable rapid resumption of *C. fimbriatum* growth when environmental conditions become more favorable, hence, when ethylene levels and/or signaling decreased. However, comparative studies with plants growing ex vitro are necessary to provide additional evidence regarding the potential adaptive relevance of the ethylene-induced

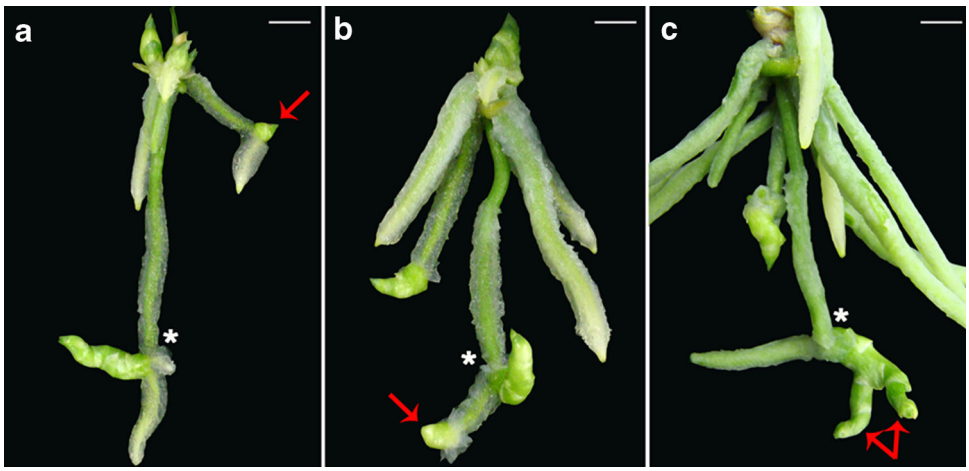

Fig. 9 Ethylene-induced bud formation in root tips of *Catasetum fimbriatum* plants: **a** Bud development in root tips of young plants near the middle of ethylene treatment period (when plants were approximately 40-day-old); *arrow* indicates a newly formed bud from an early adventitious root; **b** Morphological appearance of regenerated buds at the end of ethylene treatment (when plants were nearly 2-month-old); *red arrow* shows the initiation of a new bud from the root tip of the previous regenerated bud; **c** Morphological appearance of formed buds when previously-treated plants were 4 months old; *red arrows* indicate the shoot branching in the regenerated bud. *Asterisks* indicate the older root of each plant; note the absence of long root hairs covering the older part of this root (nearly two centimeters long), which corresponded to the portion already formed before the start of ethylene treatments. *Scale bars* 10 mm

morphogenic responses observed for *C. fimbriatum* plants growing in vitro. Further investigation regarding the ethylene mode of action during *C. fimbriatum* development will also certainly help to uncover the mechanisms behind the control of the outstanding plasticity that *Catasetum* orchids can display during their development.

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References

Baker FS, Miller CE, Repik AJ, Tolles ED (1992) Activated carbon. Kirk-Othmer Encycl Chem Technol 4:1015–1037

Benzing DH (1989) The evolution of epiphytism. In: Lüttge U (ed) Vascular plants as epiphytes: evolution and ecophysiology. Springer-Verlag, Berlin, pp 15–41

Benzing DH (1990) Vascular epiphytes: general biology and related biota. Cambridge University Press, Cambridge

Benzing DH, Ott DW, Friedman WE (1982) Roots of *Sobralia macrantha* (Orchidaceae): structure and function of the velamen-exodermis complex. Am J Bot 69:608–614

Colli S, Kerbauy GB (1993) Direct root tip conversion of *Catasetum* into protocorm-like bodies: effects of auxin and cytokinin. Plant Cell Tiss Org 33:39–44

Datta S, Kim CM, Pernas M, Pires ND, Proust H, Tam T, Vijayakumar P, Dolan L (2011) Root hairs: development, growth and evolution at the plant–soil interface. Plant Soil 346:1–14

Dycus AM, Knudson L (1957) The role of the velamen of the aerial roots of orchids. Bot Gaz 119:78–87

Franceschi VR, Nakata PA (2005) Calcium oxalate in plants: formation and function. Annu Rev Plant Biol 56:41–71

Garnett T, Conn V, Kaiser BN (2009) Root based approaches to improving nitrogen use efficiency in plants. Plant Cell Environ 32:1272–1283

Gentry AH, Dodson CH (1987) Diversity and biogeography of Neotropical vascular epiphytes. Ann Mo Bot Gard 74:205–233

Goh CJ, Kluge M (1989) Gas exchange and water relations in epiphytic orchids. In: Lüttge U (ed) Vascular plants as epiphytes: evolution and ecophysiology. Springer-Verlag, Berlin, pp 139–166

Gravendeel B, Smithson A, Slik FJW, Schuiteman A (2004) Epiphytism and pollinator specialization: drivers for orchid diversity? Philos Trans R Soc B 359:1523–1535

Gregg KB (1982) Sunlight-enhanced ethylene evolution by developing inflorescences of *Catasetum* and *Cycnoches* and its relation to female flower production. Bot Gazette 143:466–475

Karnovsky MJ (1965) A formaldehyde–glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27:137–138

Kerbauy GB (1984) Regeneration of protocorm-like bodies through in vitro culture of root tips of *Catasetum* (Orchidaceae). Z Pflanzenphysiol 113:287–291

Kerbauy GB, Colli S (1997) Increased conversion of root tip meristems of *Catasetum fimbriatum* into protocorm-like bodies mediated by ethylene. Lindleyana 12:59–63

Kerbauy GB, Estelita MEM (1996) Formation of protocorm-like bodies from sliced root apexes of *Clowesia warscewiczii*. Braz J Plant Physiol 8:157–159

Kraus JE, Monteiro WR (1989) Formation of protocorm-like bodies from root apices of *Catasetum pileatum* (Orchidaceae) cultivated in vitro. Ann Bot 64:491–498

Kress WJ (1986) The systematic distribution of vascular epiphytes: an update. Selbyana 9:2–22

Lacerda KG (1995) Amazon discovery of new species and extinction. In: Shirasawa T (ed) Brazilian Orchids. Sodo Publishing Company, Tokyo, pp 10–123

Lin Z, Zhong S, Grierson D (2009) Recent advances in ethylene research. J Exp Bot 60:3311–3336

Lüttge U (1989) Vascular epiphytes: setting the scene. In: Lüttge U (ed) Vascular plants as epiphytes: evolution and ecophysiology. Springer-Verlag, Berlin, pp 1–14

Lüttge U (2010) Ability of crassulacean acid metabolism plants to overcome interacting stresses in tropical environments. AoB Plants 2010:1–15

Moffett MW (2000) What's ''up''? A critical look at the basic terms of canopy biology. Biotropica 32:569–596

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15:473–497

Peres LEP, Kerbauy GB (1999) High cytokinin accumulation following root tip excision changes the endogenous auxin-to-cytokinin ratio during root-to-shoot conversion in *Catasetum fimbriatum* Lindl (Orchidaceae). Plant Cell Rep 18:1002–1006

Peres LEP, Amar S, Kerbauy GB, Salatino A, Zaffari GR, Mercier H (1999) Effects of auxin, cytokinin and ethylene treatments on the endogenous ethylene and auxin-to-cytokinins ratio related to direct root tip conversion of *Catasetum fimbriatum* Lindl (Orchidaceae) into buds. J Plant Physiol 155:551–555

Pierik R, Tholen D, Poorter H, Visser EJW, Voesenek LACJ (2006) The Janus face of ethylene: growth inhibition and stimulation. Trends Plant Sci 11:176–183

Pierik R, Sasidharan R, Voesenek LACJ (2007) Growth control by ethylene: adjusting phenotypes to the environment. J Plant Growth Regul 26:188–200

Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MAK (2007) Stress-induced morphogenic responses: growing out of trouble? Trends Plant Sci 12:98–105

Potters G, Pasternak TP, Guisez Y, Jansen MAK (2009) Different stresses, similar morphogenic responses: integrating a plethora of pathways. Plant Cell Environ 32:158–169

Pridgeon AM (1986) Anatomical adaptations in Orchidaceae. Lindleyana 1:90–101

Pridgeon AM (1987) The velamen and exodermis of orchid roots. In: Arditti J (ed) Orchid biology: reviews and perspectives IV. Cornell University Press, New York, pp 139–192

Rasmussen H (1986) An aspect of orchid anatomy and adaptationism. Lindleyana 1:102–107

Sánchez MI (1988) Micropropagation of *Cyrtopodium* (Orchidaceae) through root-tip culture. Lindleyana 3:93–96

Silvera K, Santiago LS, Winter K (2005) Distribution of Crassulacean acid metabolism in orchids of Panama: evidence of selection for weak and strong modes. Funct Plant Biol 32:397–407

Silvera K, Santiago LS, Cushman JC, Winter K (2009) Crassulacean acid metabolism and epiphytism linked to adaptive radiations in the Orchidaceae. Plant Physiol 149:1838–1847

Sinclair R (1990) Water relations in orchids. In: Arditti J (ed) Orchid biology: reviews and perspectives V. Timber Press, Portland, pp 63–119

Stern WL, Judd WS (2001) Comparative anatomy and systematics of *Catasetinae* (Orchidaceae). Bot J Linn Soc 136:153–178

Stern WL, Morris MW (1992) Vegetative anatomy of *Stanhopea* (Orchidaceae) with special reference to pseudobulb water-storage cells. Lindleyana 7:34–53

Strader LC, Chen GL, Bartel B (2010) Ethylene directs auxin to control root cell expansion. Plant J 64:874–884

Suzuki RM, Kerbauy GB (2006) Effects of light and ethylene on endogenous hormones and development of *Catasetum fimbriatum* (Orchidaceae). Braz J Plant Physiol 18:359–365

Suzuki RM, Kerbauy GB, Zaffari GR (2004) Endogenous hormonal levels and growth of dark-incubated shoots of *Catasetum fimbriatum*. J Plant Physiol 161:929–935

Suzuki RM, Kerbauy GB, Pescador R, Purgatto E, Ceccantini GCT, Ferreira WM (2010) Dark-induced hormone changes coincide with the resumption of light-inhibited shoot growth in *Catasetum fimbriatum* (Orchidaceae). J Plant Physiol 167:375–381

Thomas TD (2008) The role of activated charcoal in plant tissue culture. Biotech Adv 26:618–631

Vacin EF, Went FW (1949) Some pH changes in nutrient solutions. Bot Gaz 110:605–617

Vandenbussche F, Vaseva I, Vissenberg K, Van Der Straeten D (2012) Ethylene in vegetative development: a tale with a riddle. New Phytol 194:895–909

Withner CL (1959) The orchids: a scientific survey. The Ronald Press Company, New York

Yan N, Hu H, Huang J, Xu K, Wang H, Zhou Z (2006) Micropropagation of *Cypripedium flavum* through multiple shoots of seedlings derived from mature seeds. Plant Cell Tissue Organ Cult 84:113–117

Yoo SD, Cho Y, Sheen J (2009) Emerging connections in the ethylene signaling network. Trends Plant Sci 14:270–279

Zimmerman JK (1990) Role of pseudobulbs in growth and flowering of *Catasetum viridiflavum* (Orchidaceae). Am J Bot 77:533–542

Zimmerman JK (1991) Ecological correlates of labile sex expression in the orchid *Catasetum viridiflavum*. Ecology 72:597–608

Zotz G, Hietz P (2001) The physiological ecology of vascular epiphytes: current knowledge, open questions. J Exp Bot 52:2067–2078

Zotz G, Winter K (1994) Annual carbon balance and nitrogen-use efficiency in tropical C_3 and CAM epiphytes. New Phytol 126:481–492