Effects of light and ethylene on endogenous hormones and development of *Catasetum fimbriatum* (Orchidaceae)

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This study attempted to clarify the effects of dark, light and ethylene on plant growth and endogenous levels of indole-3-acetic acid (IAA), cytokinins and abscisic acid in *Catasetum fimbriatum*. Dark-incubation fully inhibited root and pseudobulb formation as well as leaf growth, but favored shoot elongation. The results of continuous and active growth in dark-incubated shoots (stolons) were induced by strong apical meristem sink activity and by the significantly increased levels of cytokinins in shoots. In fact, shoot length, cytokinin and IAA levels in dark-incubated shoots were about twice as great as for those grown under light conditions. Moreover, the total cytokinin level in shoots of *C. fimbriatum* under light conditions without ethylene was significantly higher than that found in roots. High levels of cytokinins in dark-grown stolons may be closely related to the absence of roots in *C. fimbriatum*. Under light conditions, the increased IAA level in shoots is mediated by ethylene. However, ethylene caused a significant increase of cytokinins in roots of light-treated plants, which may be involved in the retardation of root growth. Since the difference of cytokinins in shoots between ethylene-treated and non-treated plants under light conditions is small, it is concluded that the marked inhibition of leaf growth in ethylene-treated plants can be attributed to ethylene. Zeatin and zeatin riboside are the major cytokinins in *C. fimbriatum* regardless of the light conditions, ethylene treatment or organ types.

Key words: auxin, cytokinins, etiolation, plant growth, plant hormones

Efeitos da luz e etileno sobre hormônios endógenos e o desenvolvimento de plantas de *Catasetum fimbriatum* (Orchidaceae): Neste estudo, procurou-se esclarecer os efeitos do escuro, da luz e do etileno no crescimento vegetal e nos teores endógenos de ácido indolil acético (AIA), citocininas e ácido abscísico em *Catasetum fimbriatum*. A incubação no escuro inibiu completamente a formação de pseudobulbos e raízes, e reduziu drasticamente o crescimento foliar, porém estimulou substancialmente o alongamento caulinar. Os resultados do crescimento ativo e contínuo dos caules incubados no escuro (estolões) dessa orquídea foram induzidos por um aumento intenso da atividade de dreno do meristema apical e pelo aumento significativo nas quantidades de citocininas nos caules. De fato, o comprimento caulinar e os teores de citocininas e de AIA foram mais de duas vezes maior nos caules incubados no escuro do que naqueles crescidos à luz. Além disso, é importante notar que o teor de citocininas totais nos caules de *C. fimbriatum* crescidos sem etileno, no claro, foi significativamente maior do que o encontrado nas raízes. Altas concentrações de citocininas nos estolões crescidos no escuro podem estar intimamente relacionadas à ausência de raízes em *C. fimbriatum*. À luz, o aumento dos teores de AIA nos caules foi mediado pelo etileno. Entretanto, o etileno promoveu um aumento significativo de citocininas nas raízes das plantas à luz, que poderiam estar envolvidas no retardo do crescimento radicular. Uma vez que a diferença dos teores de citocininas nos caules entre plantas tratadas e não tratadas com etileno, à luz, foi pequena, conclui-se que a inibição marcante do crescimento foliar em plantas tratadas com etileno pode ser atribuída ao etileno exógeno. Zeatina e zeatina ribosídica foram as principais citocininas em *C. fimbriatum*, independentemente de luz, etileno e órgão vegetal.

Palavras-chave: auxina, citocininas, crescimento vegetal, estiolamento, hormônios vegetais

INTRODUCTION

Light leads plants to acquire a shoot morphology designed to carry out photosynthesis, while dark maximizes cell elongation, and reduces leaves and cotyledons, so that light conditions may be rapidly reached in order to support photoautotrophic growth (Von Arnim and Deng, 1996). Light and hormones control many important processes in plant development (Chory, 1993; Kraepiel et al., 2001), such as the cell cycle (Symons and Reid, 2003) and cell division in apical meristems (Von Arnim and Deng, 1996). However, the relationship between light and plant hormones is not completely understood (Kraepiel and Miginiac, 1997).

Catasetum fimbriatum (Morren) Lindl. is an epiphytic orchid, which, when grown aseptically in the dark, forms discoloured stolon-like structures with continuous longitudinal growth consisting of nodes, internodes and lateral buds covered by reduced scale-like leaves (Kerbauy et al., 1995). Recent evidence indicates that gibberellins are one of the most important hormones related to dark etiolation in this orchid (Suzuki et al., 2004). Light-grown plants, on the other hand, show limited longitudinal shoot growth, rapidly forming short and round pseudobulbs and normal green leaves (Suzuki and Kerbauy, 1999). Several effects of ethylene on shoot growth (Cary et al., 1995), cell division (Kazama et al., 2004) and hormone levels (Peres et al., 1999) have been reported. Up to now, experiments conducted to explore plant growth and development controlled by internal cues, such as hormonal balance, and by external factors have not, unfortunately, yielded conclusive information.

This study aimed to evaluate the roles of ethylene and light with regard to some morphological features, as well as endogenous levels of cytokinins, indole-3-acetic acid (IAA), and abscisic acid (ABA) in plants of *C. fimbriatum*.

MATERIAL AND METHODS

Plant material: Plants of *Catasetum fimbriatum* (Morren) Lindl. (Orchidaceae) (clone CFC1) were obtained by the etiolated shoot micropropagation technique (Kerbauy et al., 1995). Nodal segments of etiolated shoots of about 5 mm long were incubated in the dark and in the light in Vacin and Went medium (1949) modified by substituting $Fe_2(C_4H_4O_6)$ for Fe-EDTA, and supplemented with micronutrients of Murashige and Skoog (1962). The pH of medium was adjusted to 5.8 before adding 0.2% Phytagel®, and autoclaved for 15 min at 120°C. 2-chloroethyl-phosphonic acid (CEPA), an ethylene-releasing compound, was filter sterilized with Millex® HV (0.45 µm) and added to the medium at two concentrations (4.20 µM and 13.84 µM) when its temperature reached 40°C. Preliminary experiments indicated that these concentrations are, respectively, the most appropriate to evaluate the influence of ethylene on endogenous hormone levels, and the effects on growth patterns and development.

Each treatment consisted of three Erlenmeyer flasks (250 mL) containing 80 mL of culture medium and 15 nodal segments, and sealed with rubber stoppers. Cultures were incubated at 25 ± 2°C in the dark or under a 16-h photoperiod using fluorescent bulbs at 40 µmol m^{-2} s^{-1} for 60 d. This method was used to evaluate the effects of exogenous ethylene and hormone quantification in shoots and roots. The length and dry mass of these organs were recorded on the 60th d of culture. For determination of shoot growth, only pseudobulb sizes were measured, and leaves were not considered. Growth analyses were carried out with 45 plants (three flasks with 15 plants each) and values were expressed as the mean ± standard error.

Determination of endogenous hormone levels: The endogenous levels of IAA, ABA, zeatin (Z), zeatin riboside ([9R]Z), isopentenyladenine (IP), and isopentenyladenosine ([9R]IP) were measured using 1 g of fresh tissue. The immunoenzymatic method was performed according to Maldiney et al. (1986) with some modifications (Peres et al., 1997). This method allowed the determination of the three hormonal classes in the same extract. Freeze-dried powdered tissues were stirred and extracted in 10 mL of cold 80% (v/v) aqueous methanol containing butylhydroxytoluene (0.18 mM) as an antioxidant, for 60 h at 4°C in darkness. Tritiated radiolabelled standards ([^{3}H]IAA, Amersham®, [^{3}H]ABA, Amersham® and [^{3}H]Z, Isotope Laboratory®, with a specific activity of 999 GBq $mmol^{-1}$, 2.37 TBq $mmol^{-1}$, and 0.6 TBq $mmol^{-1}$, respectively) were added to the samples for recovery estimation after purification. The methanolic extracts were filtered and then passed through Sep-Pak C18 cartridges. After filtration, eluates were reduced to

dryness in a Speed Vac concentrator (HETO® CT 110) and the residues re-dissolved in 500 mL acid water (1000 mL water + 100 µL formic acid, pH 3.0). The hormones were then separated over 80 min by HPLC (Waters® System, consisting of two 510 pumps, a 746 gradient controller, a U6K manual injector, a 486 UV absorbance detector and 746 integrator) using a reverse-phase semi-preparative µBondapack 19 x 300 mm column (Waters®), at a flow rate of 5 mL min^{-1} with a methanol/acid water gradient. The gradient consisted of 5% methanol at time zero, 30% methanol at 15 min, 45% methanol at 30 min, and 50% methanol at 50 min. In all cases, acid water was added to the methanol to obtain the corresponding 100% value. Fractions were collected at 1-min intervals and reduced to dryness in the Speed Vac concentrator quoted above. For free-IAA analysis, the corresponding fractions [retention time (RT) = 53 min] were methylated with etheric diazomethane prior to ELISA with anti-IAA-methyl antibodies. The measurements of Z (RT = 36.5), [9R]Z (RT = 42.5), IP (RT = 63), ABA (RT = 65), [9R]IP (RT = 67) were carried out by ELISA with anti [9R] Z (for Z and [9R] Z), anti [9R] IP (for IP and [9R] IP) and anti-ABA antibodies. The hormone level in each sample was measured four times and the standard error calculated.

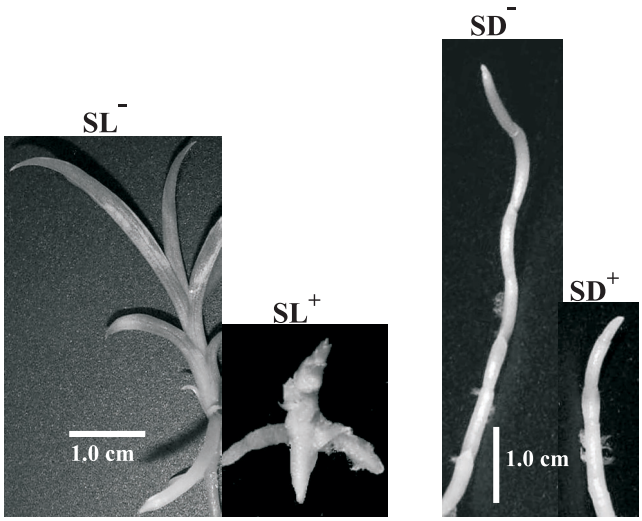

Figure 1. Phenotypes of *Catasetum fimbriatum* plants incubated for 60 d under light or dark, with or without 2-chloroethyl-phosphonic acid (at 13.84 µM), an ethylene realising compound. Dark-incubated plants did not produce any roots during the 60 d experiment. SL- = shoot under light without ethylene treatment; SL+ = shoot under light with ethylene treatment; SD- = shoot under dark without ethylene treatment; and SD+ = shoot under dark with ethylene treatment

RESULTS AND DISCUSSION

From the results in Figure 1 and Table 1, the dark treatment profoundly altered the general characteristics of the *C. fimbriatum* plants. For instance, plants grown under light for 60 d formed normal short stems, roots and leaves, while the dark-incubated individuals originated discoloured long stems (2.84-fold taller), with complete inhibition of root formation, and an increase in dry mass (1.45-fold greater). The prolonged and vigorous growth of dark-grown shoots (which looked like stolons) seems to result from the disturbance occurring in the natural source-sink competition process involving primarily the shoot apex activity and the pseudobulb formation observed in plants grown under light (Suzuki et al., 2004).

Light-incubated plants treated with ethylene led to strongly reduced etiolated shoot length (2.84-fold shorter) and leaf growth and, to some extent, retarded root growth (1.72 cm shorter). Regarding leaf inhibition, a

Table 1. Effects of 2-chloroethyl-phosphonic acid (CEPA, at 13.84 µM), an ethylene realising compound, on lengths of shoots and roots and dry matter of *Catasetum fimbriatum* plants incubated for 60 d under light and dark. Dark-incubated plants did not produce any roots during the 60 d experiment. Values are means of three replicates ± SE. SL = shoot under light; SD = shoot under dark; RL = root under light

	Lenght (cm)		Dry matter (mg)	
	Control (-)	CEPA (+)	Control (-)	CEPA (+)
SL	1.64 ± 0.35	1.66 ± 0.11	2.20 ± 0.20	2.60 ± 0.40
SD	4.66 ± 0.69	1.64 ± 0.23	3.20 ± 0.30	7.10 ± 1.00
RL	6.08 ± 0.86	4.36 ± 0.13	9.90 ± 1.60	9.40 ± 2.50

very similar phenotype to *C. fimbriatum* is frequently observed with other close genera when incubated in poorly ventilated flasks, probably due to ethylene accumulation. A recent study using mutants of tomato plants (*Lycopersicon esculentum* Mill.) that overproduce ethylene showed a substantial reduction in hypocotyl growth regardless of the light conditions and the type of light used (Carvalho and Peres, 2005). Similarly, an inhibitory effect of ethylene on root growth has also been observed in other species (Pua and Chi, 1993; Rajala et al., 2002), including *C. fimbriatum* (Kerbauy and Colli, 1997). Despite the fact that ethylene led to a substantial reduction in growth of dark-grown shoots, it had practically no effect on either shoot size or dry mass of illuminated plants (Table 1). Camp and Wickliff (1981) interpreted the reduced growth observed in ethylene/light-treated maize (*Zea mays* L.) mesocotyl as a transverse cell enlargement, due to predominantly longitudinal microfibril distribution. The very short internodes found on day 60 of light-grown plants may explain a lack of such response. The effects of ethylene on plant development usually vary according to the cell, tissue and organ types. It can for instance delay DNA synthesis and cell division in the meristems (Apelbaum and Burg, 1972), act on reorientation of microtubules and microfibrils of the cell wall (Roberts et al., 1985) and induce digestive enzymes in leaf abscission (Morre, 1968). As shown in Figure 2A, the contents of cytokinins and IAA in shoots and roots varied significantly under dark or light culture conditions. Under light, for instance, the total shoot cytokinin was about 1.7 times higher than in the roots. This result, coupled with the conspicuous accumulation of cytokinins (2.93-fold higher) detected in the dark-grown rootless shoots, indicates that *C. fimbriatum* shoots are a major organ for cytokinin production. In addition, the conspicuous cytokinin accumulation would be responsible for the absence of roots in dark-incubated plants. A stimulatory effect of a dark condition on cytokinin levels was also observed in plants of *Chenopodium rubrum* L. (Machackova et al., 1996). The increment in contents of cytokinins (2.93-fold higher) and IAA (1.89-fold higher) may be closely related to continuous and vigorous stolon growth in *C. fimbriatum* (Figure 1). The results of this paper clearly indicate that an increased level of ethylene generates a significant reduction of cytokinin in plants, leading to a decrease in shoot elongation.

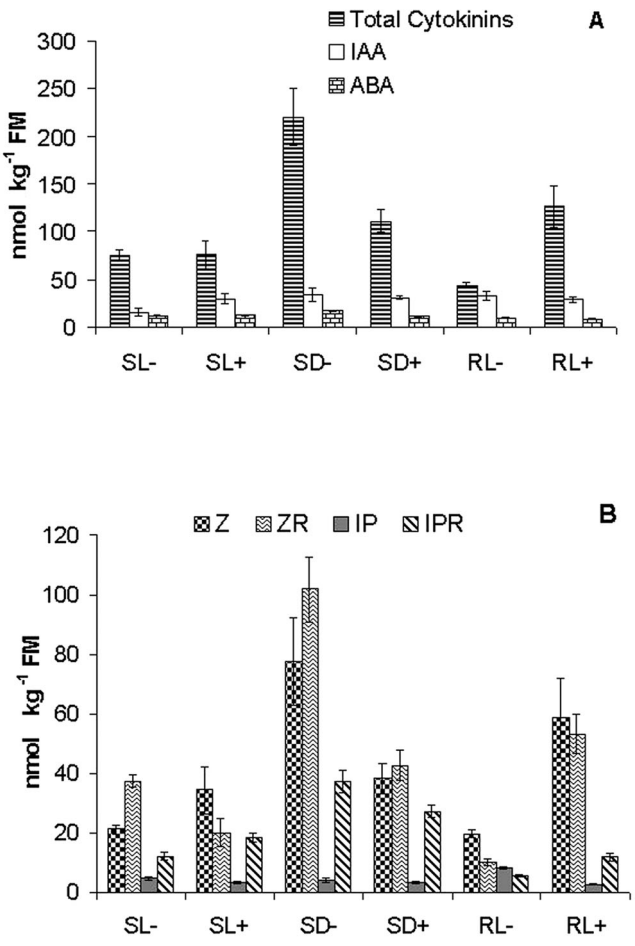

Figure 2. **A**) Total cytokinins, IAA and ABA; **B**) zeatin (Z), zeatin riboside (ZR), isopentenyl adenosine (IP) and isopentenyl adenine (IPR) in shoots and roots of *Catasetum fimbriatum* plants incubated under light or dark, with or without an ethylene realising compound (2-chloroethyl-phosphonic acid, at 4.2 μM) on the 60th d of incubation. Dark-incubated plants did not produce any roots during the 60 d experiment. Columns represent the mean of three replicates, and bars indicate standard error. SL- = shoot under light without ethylene treatment; SL+ = shoot under light with ethylene treatment; SD- = shoot under dark without ethylene treatment; SD+ = shoot under dark with ethylene treatment; RL- = root under light without ethylene treatment; and RL+ = root under light with ethylene treatment

Previous studies have established that increased amounts of cytokinins usually depress root elongation (Kerbauy and Colli, 1997; Brault and Maldiney, 1999;

Schmulling, 2002) by disturbing the apical meristem homeostasis (Massot et al., 2002) and IAA distribution in the quiescent center (Jiang and Feldman, 2003).

It is intriguing that the effects of ethylene treatments on concentrations of cytokinins and IAA in plant tissues (Figure 2A) differed significantly with growth conditions (Figure 1). In the dark-grown shoots the total level of cytokinins was halved. Under light conditions, although ethylene affected IAA levels in shoots, it did not affect cytokinin levels. In the roots of the light-grown plants, cytokinins increased by 2.9-fold compared with the dark-grown counterpart, indicating root growth inhibition (Figure 1). In fact, a significant decrease of root growth in ethylene-treated plants of *C. fimbriatum* was obtained with $AgNO_3$ and aminoethoxy-vinylglycine (AVG), two important anti-ethylenic substances (Kerbauy and Colli, 1997), accompanied by a significant drop of endogenous ethylene levels when using AVG (Peres et al., 1999).

The marked inhibitory effect of ethylene on leaf growth under light conditions (Figure 1) and the similar levels of cytokinins measured in ethylene-treated and non-treated light-grown plants allow us to hypothesize that ethylene is somehow directly involved in the process of leaf growth inhibition under light conditions.

Zeatin and zeatin riboside were clearly the two most important forms of cytokinins detected in the shoots and roots of *C. fimbriatum*, under both light regimes and ethylene treatment (Figure 2B). Furthermore, these results are in agreement with the previous findings of Peres et al. (1999) who studied root tips of this orchid species.

Regarding ABA (Figure 2A), its concentration was low and varied relatively little when compared to cytokinins or IAA levels under all experimental conditions. In contrast to what was proposed by Gazzarrini and McCourt (2001), ethylene treatment(s) in *C. fimbriatum* practically did not affect ABA concentrations.

In conclusion, light, dark and ethylene showed different marked effects on all parameters analyzed in shoots and roots. In the dark, the intense growth of shoots coincided with the significant accumulation of total endogenous cytokinins, which in fact was twice as great as under light (Figure 3). In contrast to most angiosperms, the synthesis of cytokinins by the shoots of *C. fimbriatum* plants proved to be significantly higher than by the roots.

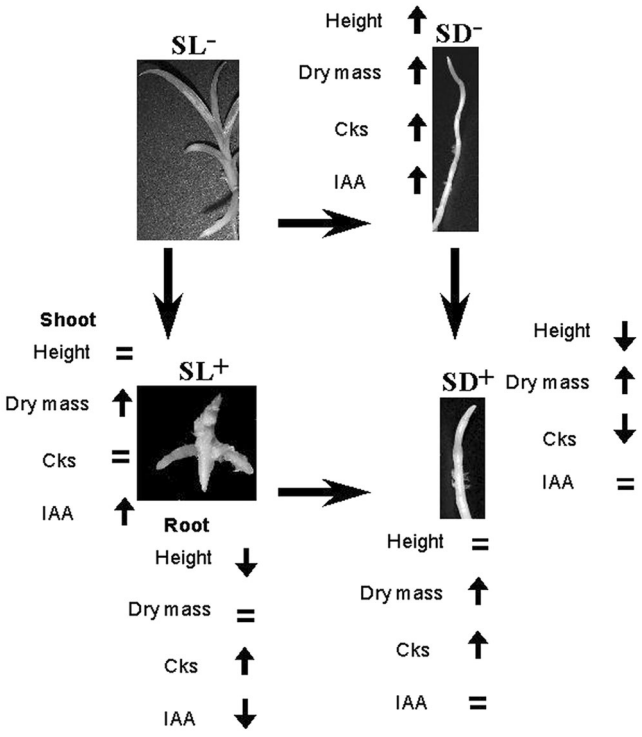

Figure 3. General effect summary of 2-chloroethylphosphonic acid (at 13.84 μM) on the developmental and hormonal status in shoot and root of *Catasetum fimbriatum* plants kept under light and dark. Larger arrows point out the experimental design for the comparisons. Cks = total cytokinins

↑ = stimulatory effect; ↓ = inhibitory effect; = = no effect

See Legend to Figure 1 for other details

The significant ethylene-induced inhibition of shoot (dark) and root (light) elongation may be caused by a reduction of longitudinal cell growth, and by increased levels of cytokinins in the shoots and roots, respectively (Table 1, Figure 2). Since total cytokinins did not practically change in the shoots of light/ethylene-treated plants, it is plausible to conclude that this growth substance was responsible for the drastic inhibition of leaf growth. It should be noted that Z and ZR were by far the two most abundant forms of cytokinins found in all experimental conditions herein assessed, but did not influence, nevertheless, the low, almost constant ABA levels.

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