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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

Lázaro E. P. Peres[1], Silvia Amar[1], Gilberto B. Kerbauy[1], Antônio Salatino[1], Gilmar R. Zaffari[2], and Helenice Mercier[1]

[1] Department of Botany, University of São Paulo, P.O. Box 11461, CEP 05422-970, São Paulo, SP, Brazil. E-Mail: lazaropp@ib.usp.br

[2] Experimental Station of Itajaí, EPAGRI, P.O. Box 277, CEP 88301-970, Itajaí, SC, Brazil

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Summary

The present investigation attempts to correlate the effect of the exogenous auxins, cytokinins and ethylene with the endogenous ethylene and auxin-to-cytokinin ratio, and with the direct root-to-bud conversion in *Catasetum fimbriatum*. Incubation of root tips with IAA and NAA strongly inhibited both root elongation and bud formation. On the other hand, treatment with IBA resulted in a conspicuous stimulation of longitudinal root growth, while bud formation was retarded. High levels of exogenous zeatin, iP and BA enhanced bud formation, and inhibited root growth. Ethylene production was measured in IAA, IBA and zeatin-treated roots. All growth regulators elicited ethylene evolution, which decreased considerably along the period of incubation. Addition of AVG to the media reduced ethylene evolution. Endogenous auxin, cytokinins, and ABA contents were determined by means of HPLC and ELISA methods in root tips of *C. fimbriatum* pre-incubated with ethylene (CEPA) and cytokinin (iP). Both exogenous substances stimulated bud formation and conduced to an endogenous IAA/Cks ratio favourable to Cks. From these results it is proposed that the stimulatory effect of exogenous Cks and ethylene on root-to-bud conversion of *C. fimbriatum* seems to be mediated by the establishment of an endogenous auxin-to-cytokinins balance favourable to shoot formation.

Key words: orchid, organogenesis, plant hormones, root meristem.

Abbreviations: [9R]iP = isopentenyladenosine; [9R]Z = zeatin riboside; ABA = abscisic acid; AVG = aminoethoxyvinylglycine; BA = benzyladenine; CEPA = 2-chloroethylphosphonic acid; Ck(s) = cytokinin(s); ELISA = enzyme linked immuno sorbent assay; HPLC = high performance liquid chromatography; IAA = indole-3-acetic acid; IBA = indole-3-butyric acid; iP = isopentenyladenine; NAA = naphthaleneacetic acid; Z = zeatin.

Introduction

Apical shoot and root meristems have been considered for a long time as poorly differentiated tissues (Esau, 1967). There is, however, consistent experimental evidence showing that meristems have specific structural/physiological identity and stability (Wareing, 1982). In this sense, the direct conversion of root tip meristems into buds was considered an uncommon organogenetic event in higher plants (Peterson, 1975; Spencer-Barreto and Duhoux, 1994), and the mecha-

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nism by which endogenous hormones affect this process is poorly known to date.

The capacity of cultured root tip segments of *Catasetum* and allied taxa to form protocorm-like bodies (PLBs) represents an authentic example of direct conversion of a root apex into a bud (Kerbauy, 1984; Kraus and Monteiro, 1989). It was previously determined that the root-to-bud conversion of *C. fimbriatum* is hastened by exogenous cytokinins (Colli and Kerbauy, 1993) and ethylene (Kerbauy and Colli, 1997) and retarded by exogenous auxins (Colli and Kerbauy, 1993). Although the role of exogenous auxin-to-cytokinin ratio on root and shoot formation is well established (Skoog and Miller, 1957), there is relatively little information on the ethylene effects in organogenetic processes (Krikorian, 1995). It is well known that the application of hormones affects the levels of endogenous hormones (Bertell and Eliansson, 1992; Etienne et al., 1993; Ribnicky et al., 1996), which should also be taken into account in the interpretation of their physiological effects.

The main purpose of this investigation was to study the effects of exogenous auxin and cytokinin on the endogenous levels of ethylene, as well as the endogenous auxin-to-cytokinin ratio in ethylene and cytokinin-treated roots related to the root-to-bud conversion process of *C. fimbriatum*.

Materials and Methods

In vitro culture

Root tips ca. 5.0 mm long, obtained from seedlings of *Catasetum fimbriatum* Lindl. (Orchidaceae), were used as explants in all experiments. Seedlings were produced asymbiotically according to Colli and Kerbauy (1993). A Vacin and Went (1949) basal medium with 2 % sucrose and modified by substituting $Fe_2(C_4H_4O_6)_3$ by 27.8 $mg \cdot L^{-1}$ Fe-EDTA, was used for root tip cultures. Erlenmeyer flasks were filled with 40 mL of culture medium. The pH was adjusted to 5.8 before adding 0.7 % agar or 0.2 % ‹Phytagel›. The media were autoclaved for 15 min at 120 °C. The cultures were maintained at 25 ± 2 °C with 16 h fluorescent light at 40–50 $\mu mol \cdot m^{-1} \cdot s^{-1}$.

Bud formation and root growth

The effects of different concentrations (0.005 to 5 $\mu mol \cdot L^{-1}$) of three auxins (IAA, IBA and NAA) and three cytokinins (iP, Z and BA) on bud formation and longitudinal root growth were studied. For each treatment, three replicate flasks with ten explants were used. The percentage of buds obtained and the length of the root segments were evaluated in the 5th week of culture. The data obtained in this experiment were subjected to regression analysis.

Ethylene evolution

After observation of the effects of exogenous auxins and cytokinins on both bud formation and explant growth, 10 $\mu mol \cdot L^{-1}$ was chosen as the concentration of IAA, IBA and zeatin for the study of the effects of these growth substances on ethylene evolution. Fifteen root tips 5.0 mm long were introduced in each vial. For each treatment 10 flasks were employed. Unperforated rubber stoppers, tightly sealed with aluminum foil, were used to close the vials. The ethylene concentration in the head spaces of the vials was determined by gas chromatography using a capillary column (fused silica, 12 m × 0.25 mm, filled with Poraplot Q) and hydrogen as carrier gas at a flow rate of 1 $cm^3 \cdot min^{-1}$. The temperatures of the columns, injector and detector (FID) were 95 °C, 105 °C and 115 °C, respectively. One mL of gas samples collected from the head spaces by means of a hypodermic syringe was injected. Intermissions of 4 days between ethylene determinations were observed. Following each measurement, the flasks were open and a stream of sterile air was applied for 1 h, after which they were sealed again. In order to inhibit endogenous ethylene effect on bud formation and longitudinal explant growth, AVG, a potent inhibitor of ethylene biosynthesis (Rando, 1974), was used. After cooling the autoclaved medium to approximately 40 °C, a filter-sterilized solution of AVG was added to the final concentration of 10 $mmol \cdot L^{-1}$. The ethylene level in each treatment was measured ten times and standard errors were calculated. From these parameters, the Student's t-test was performed and comparisons between means were made.

Endogenous levels of IAA, ABA and cytokinins

The endogenous levels of IAA, ABA, Z, [9R]Z, iP and [9R]iP were measured in attached and detached root tip segments of *C. fimbriatum* using 1 g of fresh tissue (about 130 root tip segments). In order to promote bud formation, detached root tips were incubated during 10 days in a Vacin and Went (1949) medium supplemented with iP (5.0 $\mu mol \cdot L^{-1}$) or CEPA (14.0 $\mu mol \cdot L^{-1}$). The IAA, ABA and cytokinins quantification was based on an indirect ELISA with previous purification of the extracts in reversed-phase HPLC, as described elsewhere (Zaffari et al., 1998). The hormone level in each sample was measured four times and the standard errors were calculated. The experiment was repeated twice and the values were similar. Results of one experiment are given.

Results and Discussion

Effect of different auxins and cytokinins on root tip conversion and root growth

A marked decrease in bud formation was observed at concentrations higher than 0.1 $\mu mol \cdot L^{-1}$ of either NAA, IBA or IAA (Fig. 1A). On the other hand, higher levels of BA, Z or iP promoted a substantial increase in root tip conversion (Fig. 1 B). Among these cytokinins, BA and zeatin showed the strongest effects on bud formation, while the effect of iP was clearly weaker. A significantly inhibitory effect of three auxins (IAA, IBA and 2,4-D) and a stimulatory effect of BA on root bud formation in *C. fimbriatum* were later reported by Colli and Kerbauy (1993). In the present study, a possible explanation for the comparatively lower effect of iP on bud formation could be the preference of cytokinin oxidases for iP and its riboside form as substrate (Hare & Van Staden, 1994).

There was a stimulatory effect of IBA in the growth of the root segments, and a marked limiting effect of IAA, NAA and all cytokinins at concentrations above 0.1 $\mu mol \cdot L^{-1}$ (Figs. 1C, D). In addition, it was observed that high levels (2.0 and 3.0 $\mu mol \cdot L^{-1}$) of IAA and NAA, but not IBA, induced a conspicuous process of callus formation on the root tips, followed by peeling of the epidermal cell layers of the elongation region (data not shown). Clearly, both callus formation (promoted by IAA and NAA) and direct root-to-bud conversion (promoted by Cks) were inversely proportional to root elongation. The absence of both processes in IBA treatments could account for its beneficial effects in the growth of orchid

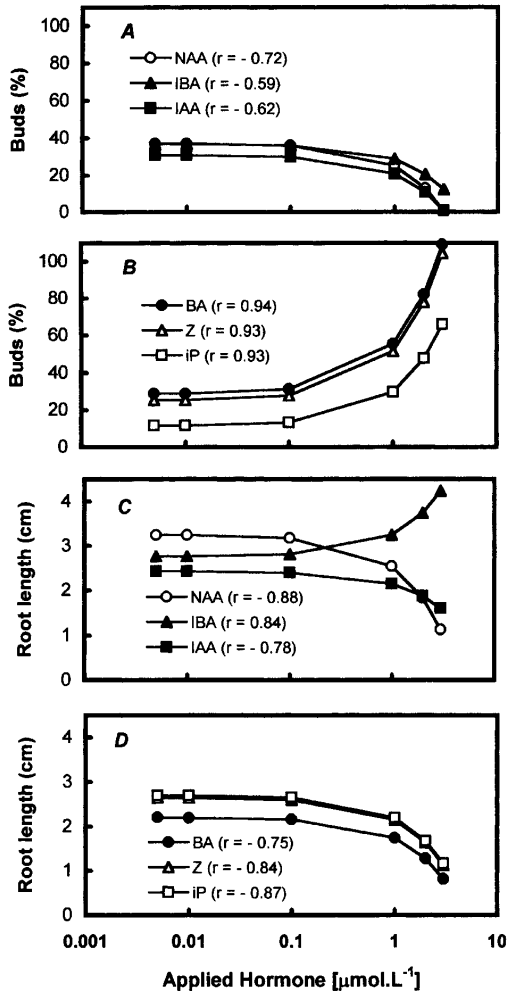

Fig. 1: Effects of several auxins and cytokinins on bud formation (A and B) and length (C and D) of root tip segments of *C. fimbriatum* following five weeks of culture. The correlation coefficients were calculated considering the levels of auxins and cytokinins, as well as root tip conversion and root length.

root segments, which was also observed with *Oncidium varicosum* and *Cattleya* (Kerbauy, 1988, 1991).

Effect of exogenous auxins and cytokinin on ethylene evolution and root tip conversion

A pronounced ethylene production was observed, 4 days after root incubation whether growth substances (IAA, IBA or zeatin) were present in the medium or not. However, as a rule, ethylene evolution was substantially lower at later measurements (Table 1). As an exception, the ethylene evolution on the 8th day in control roots without AVG was still high, although it had decreased by the 12th day of incubation. The relatively high levels of ethylene observed on the 4th day of culture seems to reflect the physical stress caused by the explant manipulation, which can induce ethylene evolution (Yang & Hoffman, 1984). Considering the amounts found in roots incubated in the hormone-free medium (control), the presence of IAA, IBA and zeatin in the media clearly conduced to a general enhancement in ethylene evolution (Table 1). However, the high ethylene levels found in control roots without AVG in the 8th day of incubation made this treatment significantly higher (P = 0.05) than IAA, IBA and zeatin-treated roots. It is interesting to note that in the 4th day of culture, IBA application seems to have a weaker affect on ethylene evolution, although it was significantly (P = 0.05) higher than the control (Table. 1). Following a treatment with AVG, ethylene production was reduced at day 4 in all treatments, decreasing even further thereafter in the IAA-treated roots, but not significantly (P = 0.05) in IBA and zeatin treatments (Table 1). The effects of auxins (Abeles, 1973) and cytokinins (Bertell and Eliasson, 1992; Cary et al., 1995) on ethylene evolution are well known. It is also well established that both the stimulatory effect of auxin and cytokinins and the inhibitory effect of AVG in ethylene production are at the level of the ACC sinthase enzyme, although they may exert their effects by different biochemical mechanisms (McKeon et al., 1995).

Data on Table 1 show that bud formation occurred at the 12th day of incubation in control roots, but not in control roots plus AVG. This result apparently corroborates the stimulatory effect of exogenous ethylene on bud formation observed by Kerbauy and Colli, 1997. However, despite the putative inhibiting effect of AVG in ethylene synthesis, bud formation was observed either with or without this substance in zeatin-treated roots (Table 1). Moreover, the high amounts of ethylene observed in IAA-treated roots (without AVG) and the reduced levels in the presence of AVG also did not influence bud formation (Table 1). These results, together with those presented in Figs. 1 A and 1 B, strongly suggest that the presence of high Cks or low auxins levels was more determinant to bud formation than ethylene levels. From these considerations, it follows that the previous reported high stimulatory effect of ethylene, ACC and CEPA on bud formation (Kerbauy & Colli, 1997) should be an indirect consequence of their high levels, thus altering the endogenous auxin-to-cytokinins ratio of the *Catasetum* root explants.

Endogenous auxin-to-cytokinin ratio in cytokinins and ethylene-treated roots

Studies performed in this laboratory with *Catasetum* and allied genera have shown that while some isolated root tip segments can be converted into buds in hormone-free media, attached roots are unable to form buds (Kerbauy, 1984), even in the presence of relatively high levels of cytokinins (Colli and Kerbauy, 1993). To examine an endogenous hormonal status possibly associated to the root-to-bud conversion, we determinated the endogenous levels of IAA, ABA and Cks in attached root (0 % of bud formation) and root tip segments

Table 1: Effects of 10 $\mu mol \cdot L^{-1}$ IAA, IBA and zeatin on ethylene production ($\mu L \cdot g^{-1} \cdot FW^{-1}$) and bud formation (%) from root tip segments of *C. fimbriatum* on different days of culture in medium with 10 $mmol \cdot L^{-1}$ AVG (AVG+) or without AVG (AVG−). Bud formation occurred only by the 12th day. The values represent the average ± standard error for ethylene production (n = 10) and bud formation (n = 10 flasks with 15 roots each).

Treatments	Ethylene production						Bud formation	
	4th day		8th day		12th day		12th day	
	AVG (−)	AVG (+)	AVG (−)	AVG (+)	AVG (−)	AVG (+)	AVG (−)	AVG (+)
Control	12.04± 3.73	1.34±0.61	15.93±2.30	0.24±0.13	0.57±0.15	0.44±0.18	3.00±0.74	0
IAA	54.69±17.80	6.16±0.60	11.91±0.75	4.68±0.47	8.02±0.39	1.97±0.43	0	0
IBA	17.91± 2.56	8.11±0.68	9.56±0.45	10.26±0.63	7.87±0.73	6.14±0.43	0	0
Zeatin	26.08± 5.36	8.46±0.31	7.03±0.91	8.58±0.32	3.77±0.66	2.89±0.37	2.71±0.79	3.71±0.32

incubated in iP or CEPA-added media (Table 2), which presented 100 % of root-to-bud conversion after the 40th day of incubation (data not shown).

Regardless of prior incubation with iP or CEPA, detached roots presented high endogenous levels of cytokinin ribosides ([9R]Z and [9R]iP) when compared to attached ones (Table 2). Cytokinin ribosides have been thought of as transport forms (Letham and Palni, 1983), and their enhancement in cultured root segments was attributed to an interruption in the export to shoots (Van Staden and Smith, 1978). Root tip segments showed a reduced content of free cytokinins (Z and iP) compared to attached ones. However, the endogenous iP, and also its riboside form was substantially high in iP- treated roots, as expected (Table 2). Total Cks content in CEPA and iP-treated root segments was significantly higher than in attached roots ($P = 0.05$ and $P < 0.001$, respectively). Although the level of IAA in the attached roots and iP-treated segments were essentially the same, CEPA-treated segments showed a reduced IAA amount (Table 2). A relationship between ethylene treatments and the reduction in the endogenous IAA levels was also observed in non-orchidaceous roots (Bertell and Eliasson, 1992), the disturbance in the auxin polar transport being related to this process (Suttle, 1988). As for the ABA content, if on one hand their level was the same in attached roots and CEPA-treated ones, on the other hand it was very low in the iP-treated roots (Table 2).

Table 2: Endogenous levels ($pmol \cdot g^{-1} \cdot FW^{-1}$) of IAA, ABA and four cytokinins in root tip segments of *C. fimbriatum* at the moment of explantation (attached roots), and at the 10th day of incubation with CEPA (14.0 $\mu mol \cdot L^{-1}$) and iP (5.0 $\mu mol \cdot L^{-1}$). The values represent the average ± standard error (n = 4). Cks = Z + [9R]Z + iP + [9R]iP.

Measured hormones	Attached roots	Root tip segments	
		CEPA-treated	iP-treated
Z	18.57±3.03	0.72±0.09	2.69± 0.18
[9R]Z	29.02±2.79	13.44±0.65	42.93± 8.49
iP	16.05±1.39	4.42±0.50	162.92±26.36
[9R]iP	10.45±1.28	70.63±4.14	343.20±41.00
CKs	74.09±4.90	89.21±3.11	551.75±43.89
IAA	66.57±7.87	28.92±4.62	56.82±10.41
ABA	52.15±1.64	55.72±0.79	10.50± 0.84
IAA/CKs	0.90	0.32	0.10

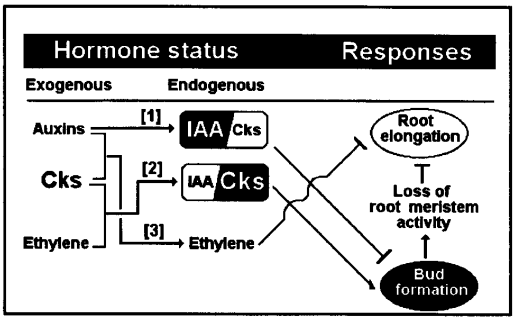

Fig. 2: A hypothesis which might account for the effects of exogenous auxin, cytokinins (Cks) and ethylene on the endogenous IAA/Cks ratio and ethylene levels and their consequence on root elongation and bud formation in *C. fimbriatum*. Rectangles represent AIA/Cks ratios which are alternately favourable to each hormone showed in black background color. Arrows indicate stimulatory effects, while «T» symbols denote inhibitory effects. The numbers [1] to [3] represent interactions that are described in the text.

The low levels of IAA observed in the CEPA-treated roots and the high level of Cks in iP-treated ones conduced to IAA/Cks ratios considerably low in these two treatments, when compared to attached roots (Table 2). Having in mind that both ethylene (Kerbauy and Colli, 1997) and Cks (Fig. 1 B) exhibit stimulatory effects on root tip conversion and that attached roots of *C. fimbriatum* are unable to form buds (Kerbauy, 1984), it seems reasonable to assume that the root-to-bud conversion process in *C. fimbriatum* is mainly controlled by the establishment of an endogenous IAA/Cks ratio favourable to Cks.

Concluding remarks

The view that emerges from the present results and others reported in the literature point out to the occurrence of an integrated hormonal control of the process of root tip conversion and root elongation in *Catasetum* (Fig. 2). The effects of exogenous auxins (Fig. 1 A), Cks (Fig. 1 B) and ethylene (Kerbauy and Colli, 1997) on bud formation appears to be mediated by alterations in the endogenous IAA/Cks balance. As

to the root elongation, the data shown in Table 1 suggest that the effect of both auxins (IAA and NAA, Fig. 1 C) and Cks (Fig. 1 D) are mediated by the promotion of ethylene production (Fig. 2, [3]), which strongly inhibits root growth (Kerbauy and Colli, 1997). Moreover, the loss of root meristem activity during the root-to-bud conversion (Kraus and Monteiro, 1989) caused by Cks and ethylene treatments could lead to a lack of cell supplementation for the elongation zone, thus inhibiting root growth. The data presented in Table 2 suggest that exogenous Cks and ethylene would promote bud formation as consequence of endogenous IAA lowering and Cks enhancement, both reflecting in an endogenous IAA/Cks ratio favourable to Cks (Fig. 2, [2]). On the other hand, auxins (IAA, NAA and IBA) seem to inhibit this process by the establishment of the inverse IAA/Cks ratio (Fig. 2, [1]). Further works involving the study of the uncommon root-to-bud conversion of *C. fimbriatum* could supply more evidences for the above hypothesis and account for a better understanding of the role of exogenous growth regulators on this and other organogenetic models.

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