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BRIEF COMMUNICATION

Abscisic acid and auxin accumulation in *Catasetum fimbriatum* roots growing *in vitro* with high sucrose and mannitol content

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Abstract

Endogenous contents of indolyl-3-acetic acid (IAA) and abscisic acid (ABA) were quantified in excised roots of *Catasetum fimbriatum* (*Orchidaceae*) cultured *in vitro* on solidified Vacin and Went medium with 1, 2, 4, 6, 8 and 10 % sucrose, as well as 2 % sucrose plus mannitol. Maximum root growth was observed in media with 4 % sucrose and 2 % sucrose plus 2.2 % mannitol, suggesting that a moderate water or osmotic stress promotes orchid root growth. Contents of both ABA and IAA increased in parallel to increasing sucrose concentration and a correlation between root elongation and the ABA/IAA ratio was observed. Incubating isolated *C. fimbriatum* roots with radiolabeled tryptophan, we showed an accumulation of IAA and its conjugates.

Additional key words: hormone interactions, root elongation, osmotic stress.

Aerial roots of epiphytic orchids are permanently exposed to heat and wind, thus suffering low water potentials (Benzing 1996, Sanford 1974), which does not seem to be a serious limitation for their growth. Further, the use of controlled water stress conditions to improve adventitious root formation and growth is a common practice used by some orchid growers (Kerbauy 1993). The mechanisms controlling epiphytic orchid root growth, and specifically under water deficit stress, are unknown. The accumulation of abscisic acid (ABA) has been shown to be required for the maintenance of maize primary root elongation at low water potentials (Sharp *et al.* 1994, Spollen *et al.* 2000). Indole-3-acetic acid (IAA) content in maize roots also increases under water stress (Ribaut and Pilet 1994) and both IAA and ABA can stimulate or inhibit root growth depending on the exogenous concentration applied and the endogenous status of each hormone class (Pilet and Saugy 1987). It was also demonstrated that the inhibitory effect of excess auxin in different organs is linked to ABA accumulation (Grossmann *et al.* 1996). On the other hand, although it is well documented that ABA can be produced and accumulated in roots (Walton *et al.* 1976, Zhang and Davies 1989, Saab *et al.* 1990, Ribaut and Pilet 1991), these organs are not considered the main sites for IAA biosynthesis (Feldman 1980, Ribaut *et al.* 1993, Ljung *et al.* 2005). Thus, the goals of the present study were: *1*) to determinate the endogenous contents of IAA and ABA in orchid roots grown *in vitro* under different sucrose and/or mannitol concentrations; and *2*) to perform a preliminary study of the orchid root ability for *de novo* auxin synthesis.

Asymbiotically-grown *Catasetum fimbriatum* Lindl (*Orchidaceae*) seedlings were isolated and propagated vegetatively *in vitro* from etiolated shoot segments generated in the dark, as described by Colli and Kerbauy (1993). The plant populations produced from each isolated seedling constitute genetically homogenous genotypes of *C. fimbriatum* named CFC1, CFC2 and so on. These materials have been maintained in our laboratory since 1993 without callus stage, in hormone free-media and using meristematic explants, so as to

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Abbreviations: ABA - abscisic acid; ELISA - enzyme linked immunosorbent assay; HPLC - high performance liquid chromatography; IAA - indole-3-acetic acid.
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ensure their genetic integrity. Root tips *ca*. 5 mm long, obtained from seedlings of CFC1, were used as explants. A hormone free Vacin and Went (1949) basal medium modified by substituting $Fe_2(C_4H_4O_6)_3$ by 27.8 mg dm^{-3} Fe-EDTA and with the addition of Murashige and Skoog (1962) micronutrients, as well as sucrose and/or mannitol according to the treatments (see below), was used for root tip cultures. The pH of the medium was adjusted to 5.8 before adding 0.7 % agar, and the medium was autoclaved for 15 min at 120 ºC. Cultures were maintained at temperature of 25 ± 2 ºC and 16-h photoperiod with an irradiance of 40 µmol m^{-1} s^{-1}.

The sucrose concentrations were 0, 1, 2, 4, 6, 8 and 10 %, and those of mannitol were 1.08, 2.2 and 3.3 % to obtain similar osmotic potential of the media. To avoid starvation stress, all mannitol treatments were combined with 2 % sucrose. A treatment with mannitol (1.08 %) as the only carbon source was also included. To prevent contamination of commercial mannitol and sucrose with ABA (Belefant and Fong 1989), both were re-precipitated with methanol. Each treatment comprised 10 Erlenmeyer flasks with 40 cm^3 of culture medium, and 10 explants per flask. All growth measurements and hormonal analyses were performed at the 35th day of *in vitro* culture. Root water content was measured gravimetrically.

Endogenous contents of IAA and ABA were measured using 1 g of fresh tissue by means of high performance liquid chromatography (HPLC) and an indirect enzyme linked immunosorbent assay (ELISA) with specific antibodies for IAA and ABA, as previously described (Maldiney *et al.* 1986) with some modifications (Peres *et al.* 2001, Zaffari *et al.* 1998). For the study of *de novo* IAA synthesis, root tips (30 per 6 cm^3 vial) were incubated in 0.055 µM (74 kBq cm^{-3}) of L-[5-^{3}H] tryptophan for 72 h under agitation (± 100 rpm). Alkaline hydrolysis (7 M NaOH at 100 ºC under N_2 for 3 h) was as described previously (Zaffari *et al.* 2002). All fractions collected in HPLC were methylated with ethereal diazomethane (Peres *et al.* 2001, Zaffari *et al.* 1998) before ELISA analysis.

After 35 d of culture in different sucrose concentrations, the roots of *C. fimbriatum* were greenish and without any lateral roots (data not shown). Water content in excised roots decreased concomitantly to the increase in sucrose concentrations (Table 1), which could be due to an osmotic effect of sucrose. Root elongation was strongly inhibited at 0 % sucrose and maximum root length was observed at 4 % sucrose (Table 1). A significant decrease in root length was also observed at 10 % sucrose. This suggests that a moderate water or osmotic stress (4 % sucrose) promotes orchid root elongation (Kerbauy 1993); although at higher sucrose concentration water stress may restrict plant growth (Pritchard 1994, Vinterhalter *et al.* 2006, Gonçalves and Romano 2007). Root fresh mass changed similarly to root length, however, the maximum dry mass was not at 4 % sucrose but at higher concentrations like 8 and 10 % (data not shown). The higher dry mass in treatments with high sucrose could be partially ascribed to passive accumulation of sucrose in the apoplast of roots. This is in agreement with the fact that orchid roots have a rather large apoplast due to the presence of velamen. Since we found that dry mass is not a good indicator for orchid root growth, the growth was characterized by root length (Table 1) and hormonal determinations (see below) were expressed on a fresh mass basis.

The prominent root length of *Catasetum* at 4 % sucrose (Table 1) seems to confirm previous suggestions that a moderate osmotic stress could promote orchid root elongation (Kerbauy 1993). However, as the increase in root length at 4 % sucrose, compared to the standard 2 % condition for orchids (Vacin and Went 1949, Wotavová-Novotná *et al.* 2007), could also be attributed to nutritional effects, we tested various combinations of 2 % sucrose plus mannitol as an osmoticum. As in other species (Stoop *et al.* 1996, Madhulatha *et al.* 2006), it seems that mannitol is not metabolized by *C. fimbriatum* roots, since there is no significant difference between root length at 0 % sucrose and 1.08 % mannitol (Table 1). Treatments with mannitol also presented a significant decrease in root water content, combined with increased

Table 1. Effects of sucrose and mannitol on water content, root length and contents of ABA and IAA of *C. fimbriatum* roots at 35th day of incubation in hormone-free solid media. In each column, the values followed by the same letter are not significantly different at the 5 % level according to Tukey's test.

Sucrose [%]	Mannitol [%]	Water content [%]	Root length [cm]	ABA [pmol g^{-1}(f.m.)]	IAA [pmol g^{-1}(f.m.)]
0	0	94.74a	0.75d	28.63ef	30.27cd
1	0	94.45ab	1.49cd	14.29fg	25.14cd
2	0	94.15ab	2.28bc	13.64g	9.81e
4	0	91.33c	3.75a	33.13e	12.64de
6	0	87.36e	2.87ab	51.88d	22.03d
8	0	81.33g	1.96bc	84.49b	50.59b
10	0	74.80h	1.31cd	149.69a	159.27a
0	1.08	93.31b	1.42cd	22.06f	26.37cd
2	1.08	90.67c	2.28bc	32.76e	14.13de
2	2.20	89.41d	3.07ab	36.61e	25.65cd
2	3.30	85.87f	2.98ab	74.24c	34.95c

values of root elongation (Table 1). Taken together, these results reinforce the hypothesis of a positive effect of moderate osmotic stress on *Catasetum* root elongation. However, in 2 % sucrose + 1.08 % mannitol, which represents the same osmotic potential as a 4 % sucrose, root elongation did not differ from that at 2 % sucrose, and was less than that at 4 % sucrose (Table 1). Thus, a nutritional effect of sucrose (Mingozzi and Morini 2009) cannot be ruled out.

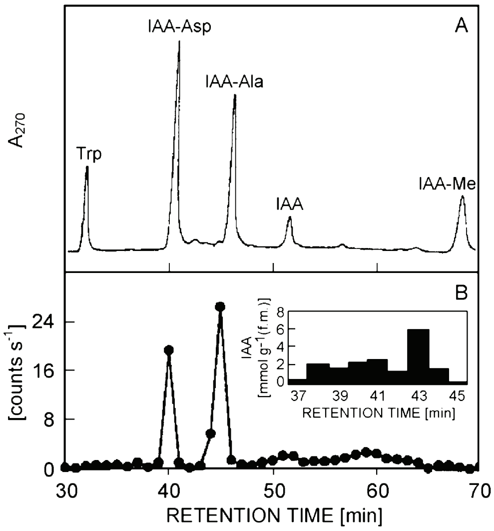

Fig. 1. Auxin production and accumulation of its conjugates in isolated root tips of *C. fimbriatum* incubated with radiolabeled tryptophan for 72 h. *A* - HPLC separation of standard substances: tryptophan (Trp), IAA-aspartate (IAA-Asp), IAA-alanine (IAA-Ala), IAA and IAA-methyl (IAA-Me). *B* - Radioactive incorporation in fractions correspondent to the HPLC separation of a representative root tip extract. The inset shows IAA contents measured in fractions with high radioactive incorporation (retention times from 37 to 45 min). These fractions were subjected to alkaline hydrolysis, using 7 M NaOH at 100 ºC under N_2 for 3 h, and methylation before quantification in ELISA using antibodies specific for IAA-Me.

In roots of *C. fimbriatum*, both IAA and ABA accumulated under a higher sucrose concentration (Table 1). As expected, there was a near perfect negative correlation (r^2 = -0.98) between relative water content (RWC) and endogenous ABA content, which supports the accuracy of the hormonal dosage method used. Since water content decreased by a maximum of 20 % (Table 1) and hormone accumulation reached up to 300 % of original values in high sucrose concentrations (Table 1); this should be attributed to a direct effect of high sucrose (Verslues *et al.* 2006) and/or osmotic stress, rather than an indirect effect of water loss (*i.e.* solute concentration). Although ABA accumulation in water stressed roots is well documented (Walton *et al.* 1976, Zhang and Davies 1989, Saab *et al.* 1990, Ribaut and Pilet 1991), there are few reports of IAA accumulation in the same conditions (Ribaut and Pilet 1994, Xin *et al.* 1997). There was no correlation between ABA or IAA accumulation and root growth since roots with almost the same length (*e.g.* at 2 and 6 % sucrose) showed different contents of IAA and ABA (Table 1). Yet, there was a trend towards accumulation of these hormones under stress conditions. This stress can be nutritional (*e.g.* 0 % sucrose and 1.08 % mannitol) or osmotic (*e.g.* 10 % sucrose), as compared to 2 % sucrose that represent the normal condition for orchid organs growth.

The accumulation of IAA in excised *C. fimbriatum* roots (Table 1) suggests a high capacity of synthesis of this hormone in an organ that is not considered its main source. Furthermore, information on biosynthesis of IAA in isolated roots is scarce (Feldman 1980, Ribaut *et al.* 1993, Ljung *et al.* 2005). Aiming to test this hypothesis, root tips of *C. fimbriatum* were treated with radioactive tryptophan, which can be an auxin precursor (Normanly *et al.* 1995). There was an accumulation of radioactivity in the retention time equivalent to auxin conjugates (Fig 1*A*). This result is in agreement with the fact that 90 % of the endogenous auxin is normally found in the conjugate form (Normanly *et al.* 1995), although with some exceptions (Patel and Thaker 2007). The alkaline hydrolysis of fractions corresponding to the conjugate accumulation and dosage with antibodies specific against IAA make it evident that the regions corresponding to the peaks contain IAA (Fig. 1*B inset*). Although we could not perform mass spectrometry to confirm the identity of peaks, this result combined with the observation that auxin accumulates in isolated roots incubated *in vitro* (Table 1) strongly suggests that *C. fimbriatum* can synthesize auxin in roots. This could be a common feature of orchid roots, as many species are known to have aerial and photosynthetic roots (Sanford 1974) and in the case of *Catasetum*, it had also been shown that its roots have the capacity of high ethylene (Peres *et al.* 1999) and cytokinin (Peres and Kerbauy 1999) production. Also, there are some orchid genera (*e.g.* *Campylocentrum*) that comprise almost shootless plants, in which roots represent the sole site for hormone synthesis (Peres *et al.* 1997).

In the present study it became clear that moderate osmotic stress promotes *Catasetum* root elongation, however, we could not find a correlation between this parameter and individual endogenous levels of IAA and ABA. An interaction between IAA and ABA in the control of root growth has been suggested before (Pilet and Saugy 1987), so we tested the hypothesis that the root elongation process could be dependent on a balance between these two hormone classes. Pooling the data presented in Table 1, we found a positive correlation (r^2 = 0.64) between the ABA/IAA ratio and root length, which is in agreement with the initial suggestion of interaction between these two hormones in controlling root growth (Pilet and Saugy 1987). Remarkably, these authors also showed that exogenous IAA and ABA can stimulate or inhibit root growth depending on the endogenous status of the respective hormone class (Pilet *et al.* 1979, Pilet and Saugy 1987). Based on the results presented here, an alternative interpretation might be that the effect of applied IAA is dependent on the endogenous

ABA level and *vice versa*. Supporting this, interactions between these two hormone classes at signal transduction level were demonstrated by some authors (Suzuki *et al.* 2001, Brady *et al.* 2003). Further work using alternative approaches and models, such as ABA and IAA double mutant analysis in *Arabidopsis* or tomato, should clarify these questions.

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