

# Regenerative Capacity of Cacti *Schlumbergera* and *Rhipsalidopsis* in Relation to Endogenous Phytohormones, Cytokinin Oxidase/Dehydrogenase, and Peroxidase Activities

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## ABSTRACT

The recalcitrant nature and increased regenerative capacity in relation to *in vitro* subcultures in two cactus genera *Rhipsalidopsis* (Easter cactus) and *Schlumbergera* (Christmas cactus) were studied by examining the endogenous concentrations of several endogenous phytohormones and enzyme activities. Leaf tissue from greenhouse-grown mother plants, *in vitro* subcultures 1 and 3, and callus tissues were analyzed and correlated with regenerative ability. The cytokinins present in the two cacti genera were mainly isopentenyl-type derivatives. The total content of isopentenyl-type cytokinins in greenhouse-grown leaves of *Rhipsalidopsis* was more than twice the amount found in

greenhouse-grown leaves of *Schlumbergera*. The total cytokinin content decreased during subculturing. Cytokinin oxidase/dehydrogenase (CKX, EC 1.4.3.18/1.5.99.12) activity increased during subculturing. In *Schlumbergera* there is no effect of subculturing on CKX and related cytokinin homeostasis. The total peroxidase (POX, EC 1.11.1.7) activity in greenhouse-grown leaves of both genera was low, and the activity increased significantly during subculturing, more specifically in the tissue of *Rhipsalidopsis*. The results clearly indicated that an enhanced auxin metabolism (biosynthesis, conjugation/deconjugation, and POX activity), in combination with an enhanced CKX activity, shifts the auxin and cytokinin pool, favoring adventitious shoot formation in *Rhipsalidopsis*, whereas the low level of POX activity, together with auxin autotrophy/conjugation, makes *Schlumbergera* more recalcitrant.

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## INTRODUCTION

Plant species differ widely in their regenerative capacity. Competent cells are those that may regenerate in response to external signals that induce a specific developmental pathway (Meins and Binns 1979). Some plants are reported to be recalcitrant, but these plants can be manipulated to regenerate (Sriskandarajah and others 1982; Gurriaran and others 1999). Recalcitrance can be a major limiting factor for *in vitro* regeneration or biotechnological modification of economically important plants.

Sriskandarajah and Serek (2004) showed that some recalcitrant cultivars of two cacti genera *Rhipsalidopsis* (Easter cactus) and *Schlumbergera* (Christmas cactus) could be manipulated to regenerate under *in vitro* conditions. A long-term period of subculturing was required to increase the regenerative capacity of explants *in vitro*, and there was a progressive improvement in regenerative capacity with successive generations of shoot explants producing adventitious shoots. On the other hand, callus explants of both cacti genera possessed high regenerative capacity. Increased regenerative ability, along with successive generations of cultures, exists in apple and hop plants (Sriskandarajah and others 1982; Bastista and others 1996; Gurriaran and others 1999). In these works, although it has been suggested that the high regenerative ability is due to a temporary change to the juvenile phase, which is normally associated with high regenerative capacity, specific mechanisms inducing this phenomenon are not understood. *In vitro* regeneration is a complex phenomenon that can be influenced by a number of genetic and environmental factors. Some reports indicate that the recalcitrant nature of plant protoplasts is related to oxidative stress (Papadakis and Roubelakis-Angelakis 1999, 2002; Papadakis and others 2001). It is well known that cytokinins and auxins play key roles in controlling shoot development and it is possible that endogenous changes of these plant hormones in explants may contribute to their increased regenerative capacity. In tissue culture, concentrations of active phytohormones within explants are influenced by both exogenous factors and endogenous metabolism (Krikorian 1995; Auer

and others 1999; Benson 2000; Motyka and others 1996, 2003). Although it is possible to modify the regenerative capacity in some plants by altering cytokinin homeostasis, this may not be applicable to all species (Boiten and others 2004). Cytokinin biosynthesis occurs mainly via the corresponding ribotides, whereas cytokinin degradation is mediated by cytokinin oxidase/dehydrogenase (CKX, EC 1.4.3.18/1.5.99.12). The biochemical pathways controlling cytokinin homeostasis could be affected by tissue culture conditions, explant history, morphology, genotype, and many other factors (Krikorian 1995; Auer and others 1999).

Auer and others (1999) demonstrated that the content of endogenous isoprenoid cytokinins, especially  $N^6$ -( $\Delta^2$ -isopentenyl) adenine (iP) and  $N^6$ -( $\Delta^2$ -isopentenyl) adenosine (iP R), increased along with an enhancement of CKX activity in early shoot development in a highly organogenic line of *Petunia*. Therefore, the difference in organogenic capacity could be attributed, on the one hand, to CKX activity, and on the other hand, to corresponding endogenous cytokinin levels (Kapchina-Toteva and Yakimova 1997). In addition, auxins may be degraded via oxidation. However, neither conjugates of IAA nor synthetic auxins such as 2,4-D can be destroyed by oxidase activity (Bartel 1997).

Ethylene is involved in many morphogenic responses as it can either promote or inhibit *in vitro* morphogenesis (Kumar and others 1998). Exogenous application of phytohormones stimulated ethylene production in tissue culture (Hutchinson and others 1997), and ethylene induced peroxidase (POX, EC 1.11.1.7) activity (Kato and others 2000). POXs are a group of anti-oxidative enzymes consisting of several isozymes (Kapchina-Toteva and Yakimova 1997). POX activity was reported as a marker for the rooting process in easy- and difficult-to-root species of *Gravillea* and *Protea* (Ludwig-Muller 2003), peaking during adventitious root formation in the difficult-to-root species. This indicates a strong relationship between adventitious organ formation and POX activity. Both an increase in POX activity and ethylene formation can be related to (mechanical) stress when the explants are taken from the greenhouse to *in vitro* conditions or during subsequent subculturing.

In studies with phylloclade explants of two cacti genera, *Rhipsalidopsis* and *Schlumbergera*, exogenous application of cytokinins was essential for growth or regeneration (Srisikandarajah and Serek 2004). Therefore, in the present study, we analyzed endogenous auxin, cytokinin and ACC contents, total POX and CKX activities in (1) explant tissues from the original mother plants, (2) explants from different subculture generations, and (3) calluses of *Rhipsalidopsis* (*R. gaertneri* × *R. rosea*, cultivar CB4) and *Schlumbergera* (*S. russeliana* × *S. truncata*, cultivar Olga). Results are discussed in relation to differences in regenerative ability of the explants.

## MATERIAL AND METHODS

### Plant Material

Cultivars of *Schlumbergera* (*S. russeliana* × *S. truncata*, cultivar Thor-Olga) and *Rhipsalidopsis* (*R. gaertneri* × *R. rosea*, cultivar CB4) (from Gartneriet Thoruplund, Odense, Denmark and Gartneriet PKM ApS, Odense, Denmark) were grown as mother plants in 0.5 l (10 cm square) pots in a greenhouse with 16 h light (provided by SON-T sodium lamps during winter months, 600  $\mu\text{mol m}^{-2}\text{s}^{-1}$  at plant surface) at 25°–28°C. To facilitate surface sterilization, plants were watered at the base without fertilizer and without wetting the foliage. At the start of the experiments, plants were about 1 year old and carried three to five tiers of mature phylloclades (leaves or shoots).

### Establishment and Maintenance of *In vitro* Cultures

Phylloclades (3–5 tiers from the apical phylloclade) from each cultivar were established in tissue culture (Srisikandarajah and Serek 2004). Explants were grown in MS medium (Murashige and Skoog 1962) supplemented with Staba vitamins (Staba 1969), 3.5  $\mu\text{M}$  N<sup>6</sup>-benzyladenine (BA), 2.5  $\mu\text{M}$  indole-3-butyric acid (IBA), 3% w/v sucrose, and gelled with 3 g l<sup>-1</sup> gelrite (Phytigel, Sigma) and incubated in light (17-h photoperiod of 60  $\mu\text{mol m}^{-2}\text{s}^{-1}$  provided by cool-white fluorescent tubes) at 25°–28°C.

This method induced axillary shoot growth from the phylloclade explants. When shoots developed, they were excised from the mother explants and implanted in flat-surfaced plastic containers (99 × 91 mm, Danefeld, Breeding Station, Denmark) containing fresh medium the same as above.

### Production of Shoot Subcultures and Calluses

Axillary shoots, isolated from the initial phylloclade explants, were grown in fresh shoot growth medium (see above) to multiply. New shoots were excised and subcultured every 6–15 weeks. The duration of the subculture period varied according to growth rate. At each subculture, shoots were marked and subcultured to produce successive generations (1–3 subcultures) of shoot cultures. All *in vitro* cultivation steps were conducted under light and temperature conditions as described above. Callus tissue, produced from the cut surfaces of the initial explant, was excised and subcultured separately.

### Tissue Samples for Hormone and Enzyme Analyses

Tissue samples (0.1–0.3 g FW if not otherwise stated) from the second-tier phylloclade of the greenhouse plants or from phylloclade explants from the subculture generations were taken from fresh material, flash-frozen in liquid nitrogen, and stored at –70°C until hormone and enzyme analyses were done. Data presented are the means of three independent measurements.

### Peroxidase Analysis

Activity of soluble POXs was determined according to the method of Hart and others (1971). Tissue extracts were prepared using 0.3–0.5 g material with 0.1 M sodium phosphate buffer (pH 7.0) with addition of 1mM EDTA and 10  $\mu\text{M}$  complete protease inhibitor (Roche, Mannheim, Germany). The homogenate was kept in ice for 30 min and then centrifuged at 15,000 rpm. The supernatant was assayed for POX activity. The incubation mixture contained enzyme extract, 20 mM guaiacol, 0.1 M sodium phosphate buffer and 10 mM H<sub>2</sub>O<sub>2</sub>. Absorption was measured at 470 nm for 5 min at intervals of 60 s. Enzyme activity was defined as  $\mu\text{M GDHP mg}^{-1} \text{ protein min}^{-1}$  (Guaiacol Dehydrogenation Product), using the mmolar extinction coefficient of GDHP ( $\epsilon = 26.6 \text{ mM}^{-1}\text{cm}^{-1}$ ). Protein content was determined according to Bradford (1976) using bovine serum albumin as a calibration standard.

### Determination of Cytokinin Oxidase/Dehydrogenase Activity

The method described by Motyka and others (2003) was used for extraction and partial purification of

cytokinin oxidase/dehydrogenase activity. Plant material (0.2–4.5 g fresh weight [FW]) was homogenized in cold extraction buffer (0.1 M Tris-HCl, pH 7.5). After filtration through an acid-activated polyvinylpyrrolidone column, centrifugation and removal of nucleic acids by polyethyleneimine (Polymix P, 1% v/v, pH 7.5), proteins were precipitated by 80% saturation of ammonium sulfate. Protein concentrations were determined using bovine serum albumin as a standard (Bradford 1976).

The CKX activity was determined by *in vitro* assays based on conversion of [2-<sup>3</sup>H]iP (prepared by Dr. Jan Hanuš, Isotope Laboratory, Institute of Experimental Botany AS CR, Prague, Czech Republic) to <sup>3</sup>H-adenine. The assay mixture (50 µl final volume) included 100 mM TAPS-NaOH buffer containing 75 µM 2,6-dichloroindophenol (pH 8.5), 2 µM substrate ([2-<sup>3</sup>H]iP, 7.4 TBq mol<sup>-1</sup>) and enzyme preparation equivalent to 0.1–0.25 g tissue FW. After incubation at 37°C the reaction was terminated by addition of 10 µl Na<sub>4</sub>EDTA (200 mM) and 120 µl 95% (v/v) ethanol. Separation of the substrate from the product of the enzyme reaction was achieved by a Series 200 HPLC Quaternary Pump (PerkinElmer, USA) coupled to a 235C Diode Array Detector (PerkinElmer) and a RAMONA 2000 flow-through radioactivity detector (Raytest, Germany) on the column Luna C<sub>18</sub> (2) (50 mm/4.6 mm/3 µm) connected with two C<sub>18</sub> guard cartridges as described by Blagoeva and others (2004).

### LC-(ES)-MS/MS Analysis of Cytokinins

Cytokinin (CK) extraction and purification were performed as previously described by Redig and others (1996). Deuterated cytokinins ([<sup>2</sup>H<sub>3</sub>]DHZ, [<sup>2</sup>H<sub>3</sub>]DHZR, [<sup>2</sup>H<sub>5</sub>]Z-N<sup>9</sup>-G, [<sup>2</sup>H<sub>5</sub>]Z-O-G, [<sup>2</sup>H<sub>5</sub>]ZR-O-G, [<sup>2</sup>H<sub>6</sub>]2iP, [<sup>2</sup>H<sub>6</sub>]2iPR, [<sup>2</sup>H<sub>6</sub>]2iP-G, [<sup>2</sup>H<sub>3</sub>]DHZR-P, [<sup>2</sup>H<sub>6</sub>]2iPR-P; 20 pmol for all free bases, ribosides, and ribotides; 200 pmol for all glucosides, OlChemIm Ltd., Olomouc, Czech Republic) were added as internal tracers. Cytokinins were purified combining solid phase extraction and immunoaffinity chromatography using immobilized antibodies against isoprenoid CKs (OlChemIm Ltd.). Cytokinin nucleotides were treated with alkaline phosphatase (Roche Diagnostics, Mannheim, Germany; 45 min, 37°C, pH 7).

Samples containing CKs were analyzed by micro-high performance liquid chromatography (HPLC) linked to a Quattro II mass spectrometer (Waters-Micromass, Manchester, UK) equipped with an electrospray interface (micro-LC-(ES<sup>+</sup>)-MS/MS) and a Z-spray. Samples (25 µl) were injected on a

C<sub>18</sub> reversed-phase column (Prodigy 5 µm OD83 100 A 150 × 1 mm, Phenomenex, Bester, Amstelveen, The Netherlands) and eluted using a solvent gradient (on column focusing, MeOH: 0.01M NH<sub>4</sub>OAc, 10/90 loading for 1 min, followed by a linear gradient 20/80–90/10 in 2.5 min, 90/10 for 3.5 min, isocratic flow 60 µl min<sup>-1</sup>) according to Prinsen and others (1998). The effluent was introduced into the ES source (source temp. 80°C, capillary voltage +3.5 kV, cone voltage 20 V). Quantification was done by Multiple Reaction Monitoring (MRM) of [MH]<sup>+</sup> combined with the m/z of the appropriate product ion (listed in Prinsen and others 1998). Chromatograms were processed using Masslynx 3.4 software (Waters-Micromass). Concentrations were determined following the principle of isotope dilution. Data are expressed in picomoles per gram fresh weight (pmol g<sup>-1</sup> FW).

### NICI GC-MS Analysis of IAA and ACC

Indole-3-acetic acid (IAA) and ACC were extracted by a combined solid-phase extraction procedure (Prinsen and others 2000; Smets and others 2003) using [<sup>13</sup>C<sub>6</sub>-phenyl]-IAA (100 pmol, Cambridge Isotope laboratories, Andover, Massachusetts, USA) and d<sub>4</sub>-ACC (400 pmol, Sigma, St. Louis, Missouri, USA) as internal standards. The total pool of IAA conjugates was analyzed in 1/3 of the sample after alkaline hydrolysis (Bialek and Cohen 1989). ACC-conjugates were analyzed in the remaining 1/3 fraction of the extract after dry acid hydrolysis following Chauvaux and others (1993). After alkaline hydrolysis and dry acid hydrolysis of IAA-C and ACC-C, respectively, samples were treated as free IAA and ACC. After derivatization with PFB bromide (Netting and Milborrow 1988), IAA was measured as pentafluorobenzyl (PFB)-IAA in a single gas chromatography–mass spectrometry run using a negative ion chemical ionization mode (NICI GC-MS) (NH<sub>4</sub>; HP 5890 series II coupled to a quadrupole mass spectrophotometer [Trio 2000, WATERS-Micromass, Manchester, UK]; column: 15 m BD-XLB, 0.25 mm i.d., 0.25-µm film diameter [J&W Scientific, Folsom, California, USA], gas phase He, 120°C to 240°C; 15° min<sup>-1</sup>; T<sub>inj</sub>, 250°C). PFBbis-ACC was analyzed in a separate run using the same chromatographic conditions and MS settings. The diagnostic ions used are listed in Prinsen and others (2000) and Smets and others (2003). Data are expressed in pmol per gram fresh weight (pmol g<sup>-1</sup> FW).

The total IAA pool (free IAA + IAA-conjugates) (abscissa) was plotted against the corresponding total cytokinin pool (sum of all isoprenoid

cytokinins) (ordinate) for each individual sample to obtain Skoog and Miller diagrams. This resulted in a scatter plot in which genera-related clusters could be distinguished. Skoog and Miller diagrams were constructed for greenhouse material, *in vitro* material, and calluses.

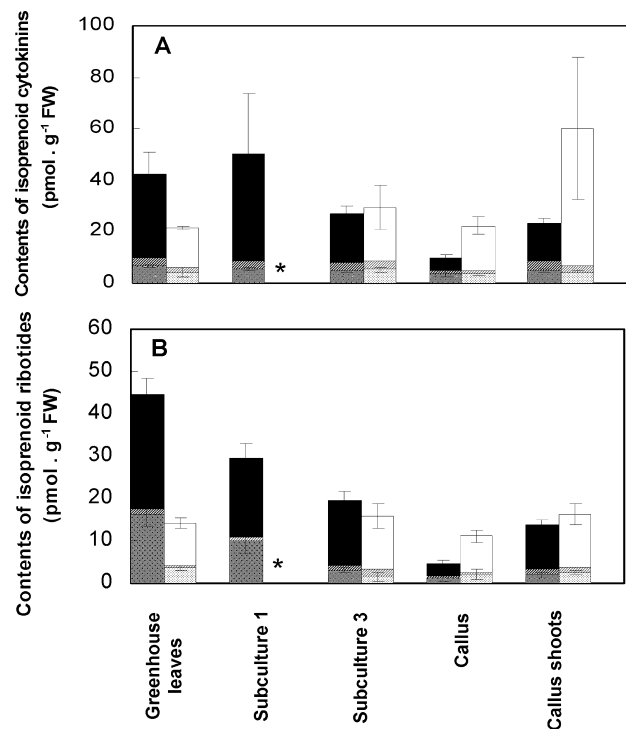
## RESULTS AND DISCUSSION

The objective of the present study was to determine if a correlation existed between endogenous phytohormone metabolism and regenerative capacity for adventitious shoot formation in phylloclade cultures of cacti *Schlumbergera* and *Rhipsalidopsis*. It is generally known that normal plant growth and development depends on the potential cross talk between cytokinins and other plant growth factors, mainly auxins. The type and concentration of phytohormones in plants are directly involved in the regenerative capacity. In particular, the ratio of auxins to cytokinins present in the culture medium plays a critical role, with higher ratios of auxins to cytokinins favoring root regeneration (Skoog and Miller 1957; Boerjan and others 1995; Liu and others 1997), lower ratios favoring shoot regeneration, and the intermediate ratios favoring callus formation.

Both cactus cultivars analyzed in this study required exogenous cytokinins for callus as well as shoot growth under *in vitro* conditions. Cultures of *Rhipsalidopsis* were considerably more regenerative than cultures of *Schlumbergera*. The initial phylloclade cultures from the greenhouse failed to produce any adventitious shoots but produced adventitious roots instead. A significant induction of shoot formation in long-term *in vitro* cultures of both cacti genera has been observed whereas root formation was inhibited in later subcultures (Sriskandarajah and Serek 2004).

### Isopentenyl-type Cytokinins Are the Major Cytokinins in *Schlumbergera* and *Rhipsalidopsis*

All isoprenoid cytokinins were screened in greenhouse leaves, in shoots from the 1st and 3rd subcultures, in callus tissue and callus shoots. To determine initial total cytokinin level, data obtained for individual cytokinins were pooled and plotted as zeatin (Z; both *cis*- and *trans*-isomers)-, dihydrozeatin (DHZ)-, and iP-type cytokinins (Figure 1A). It is evident that iP-type cytokinins predominate in both *Schlumbergera* and *Rhipsalidopsis*, whereas Z- and DHZ-type cytokinins are hardly present (<5% of the



**Figure 1.** **A.** Total pool of isoprenoid-type cytokinins in greenhouse-grown leaves during two subsequent stages of *in vitro* subculturing, callus culture, and callus shoots. Data for the endogenous cytokinins are pooled according to type. Lower bar: zeatin-type (*cis*-zeatin + *trans*-zeatin + *cis*-zeatin-*N*<sup>9</sup>-riboside + *trans*-zeatin-*N*<sup>9</sup>-riboside + zeatin-*N*<sup>9</sup>-glucoside); middle bar: dihydrozeatin-type (dihydrozeatin + dihydrozeatin-*N*<sup>9</sup>-riboside + dihydrozeatin-*N*<sup>9</sup>-glucoside); and upper bar: iP-type (iP + iPR+ iP-*N*<sup>9</sup>-glucoside). **B.** Total pool of isoprenoid ribotides. Data for the endogenous cytokinins are presented according to type. Lower bar: zeatin-type (*cis*-zeatin-*N*<sup>9</sup>-riboside-5'-monophosphate + *trans*-zeatin-*N*<sup>9</sup>-riboside-5'-monophosphate); middle bar: dihydrozeatin-*N*<sup>9</sup>-riboside-5'-monophosphate; upper bar: iPR-5'-monophosphate. Left (black bars), *Rhipsalidopsis* (cv. CB4); right (white bars), *Schlumbergera* (cv. Thor-Olga). All data are expressed as pmol g<sup>-1</sup> FW. *n* = 3; error bar: STERR; \*not analyzed.

total cytokinin pool). Thus it is assumed that Z- and DHZ-type cytokinins are of minor importance in this type of plant material.

In greenhouse leaves, the total endogenous content of isoprenoid-cytokinins in *Rhipsalidopsis* is twice as high as in *Schlumbergera* (Figure 1A). During subsequent subculturing (subculture 1 versus subculture 3) and callus culture, the total cytokinin content in *Rhipsalidopsis* tissue decreased gradually. An increase in the total cytokinin pool in callus shoots of both genera indicated that new leaves formed *in vitro* regain an active cytokinin biosynthesis and become cytokinin autotrophic.

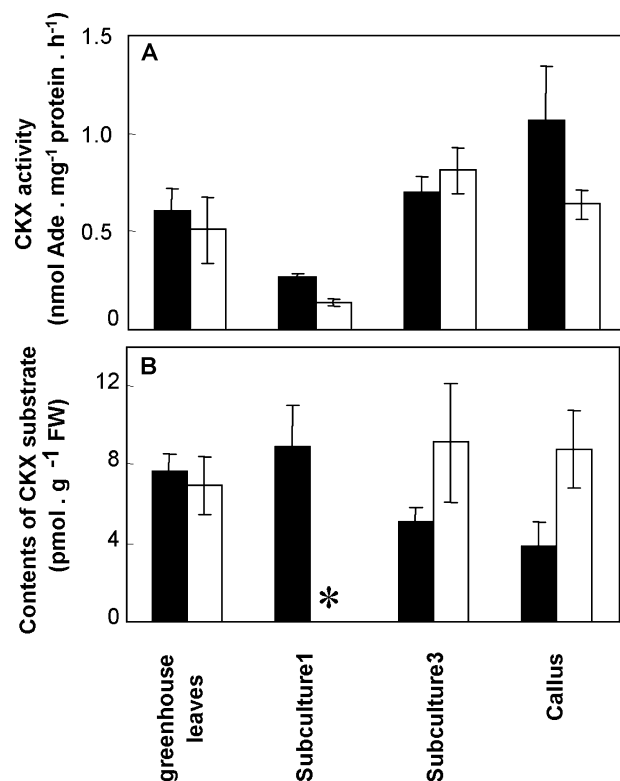
Similar to isoprenoid free bases and ribosides, iPR-monophosphate is the predominant ribotide in the total pool of isoprenoid ribotides (Figure 1B), and DHZ ribotides are present in relatively low concentrations (< 5% of the total cytokinin content). In greenhouse leaves, the total concentration of endogenous isoprenoid-ribotides in *Rhipsalidopsis* is significantly higher than the ribotide content in *Schlumbergera* reflecting a difference in cytokinin biosynthetic capacity between the two genera. The gradual decrease of ribotides during subculturing indicates cytokinin consumption or degradation, in combination with a reduced or impaired cytokinin biosynthesis capacity, which finally results in a decreased total cytokinin pool.

### Cytokinin Oxidase/Dehydrogenase Activity Increases during Subculturing

The CKX activity was analyzed in leaf tissues isolated from greenhouse-grown plants, explants from different subcultures, and calluses (Figure 2A). The CKX activity increases during subculturing in both *Rhipsalidopsis* and *Schlumbergera*. Concomitant with the induced CKX activity during subculturing is a reduction in the total amount of isoprenoid cytokinins (iP, iPR, Z, and ZR), which serve as substrates for CKX (Figure 2B). These data confirm that cytokinin degradation occurs during subculturing, and they clearly demonstrate the impact of cytokinin oxidation/dehydrogenation (degradation) by CKX(s) on the total active cytokinin pool.

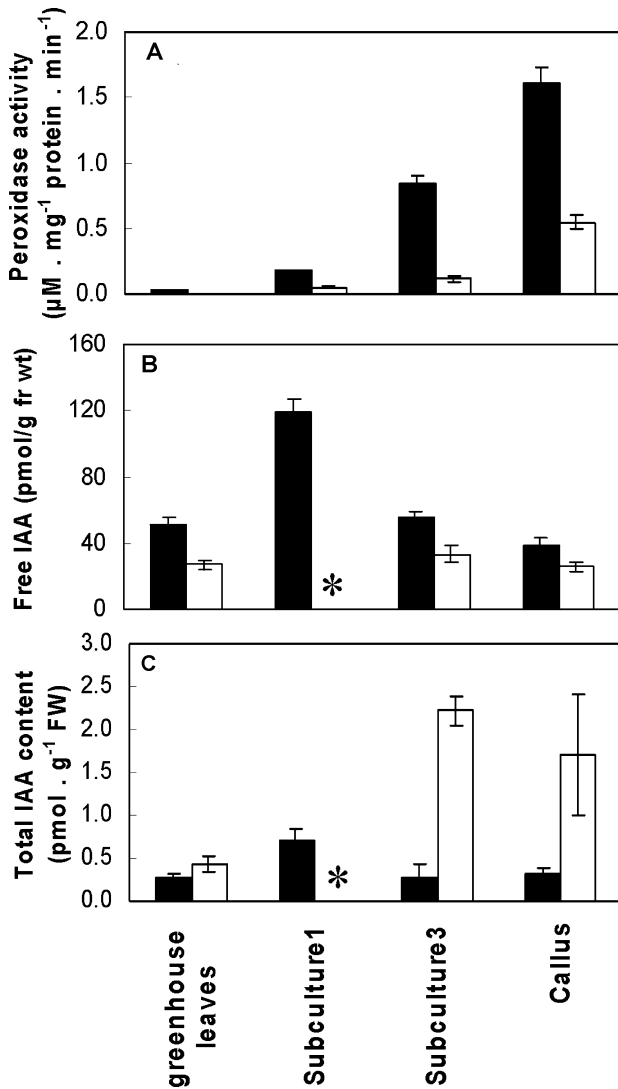
### The Peroxidase Activity Increases during Subculturing

Total POX activity was analyzed in leaf tissues from both *Rhipsalidopsis* and *Schlumbergera* (Figure 3A). For both genera, endogenous POX activity in greenhouse-grown leaves is very low ( $0.028$  and  $0.01 \mu\text{M mg}^{-1} \text{ protein min}^{-1}$  for *Rhipsalidopsis* and *Schlumbergera*, respectively). During subculturing, total POX activity increases significantly and is more than twofold higher in *Rhipsalidopsis* than in *Schlumbergera*. Concomitantly, there is a significant reduction in free IAA concentration during subculturing in *Rhipsalidopsis* (Figure 3B). Whereas the total IAA pool in *Rhipsalidopsis* remains constant during subculturing, the total IAA pool in *Schlumbergera* increases significantly to a maximum of  $2220 \pm 170 \text{ pmol g}^{-1}$  fresh weight (FW) in subculture 3 (Figure 3C). The IAA conjugates, which are storage forms insensitive to IAA oxidation, may be formed to prevent oxidation of the free base. From this pool of IAA-conjugates, active free auxins



**Figure 2.** Cytokinin oxidase/dehydrogenase (CKX) activity (A) and total amount of cytokinins susceptible to CKX catalyzed degradation (iP + iPR + zeatin + zeatin- $N^9$ -riboside) (B) in greenhouse-grown leaves, during two subsequent stages of *in vitro* subculturing and callus culture. Left (black bars), *Rhipsalidopsis* (cv. CB4); right (white bars), *Schlumbergera* (cv. Thor-Olga). All CKX data are expressed as  $\text{nmol Ade mg protein}^{-1} \text{ h}^{-1}$ ; cytokinin data are expressed as  $\text{pmol g}^{-1} \text{ FW}$ .  $n = 3$ ; error bar: STERR; \*not analyzed.

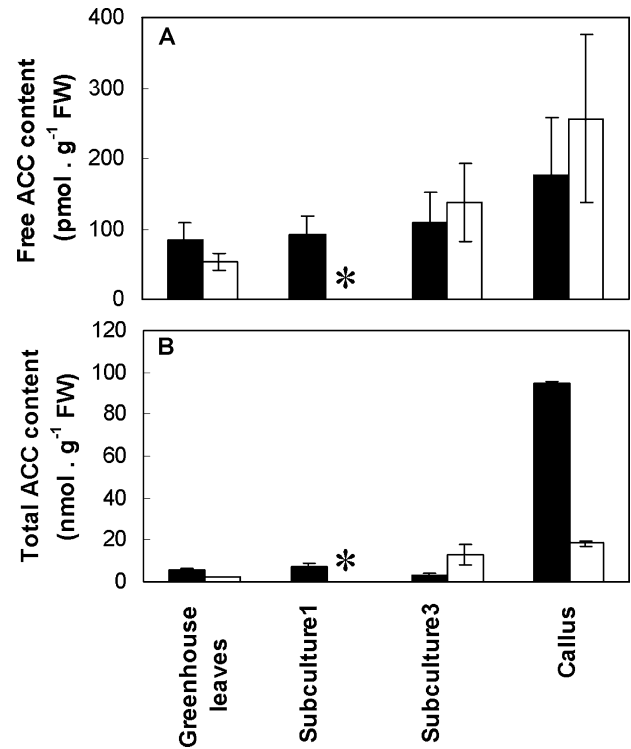
can be recruited very rapidly. The free IAA steady state is frequently controlled via conjugation/deconjugation, whereas a steady-state concentration of auxins is maintained and tightly regulated in plants. Consequently, a reduction in the free IAA pool is hardly seen (Bartel 1997), and auxin changes are reflected in the total IAA pool. In fact, the differences in free IAA levels in these cacti explants are not striking (Figure 3B). *Schlumbergera* tissue shows an active *de novo* IAA synthesis in combination with an active auxin conjugation *in vitro*, which can be seen as an increase in the total IAA pool in subculture 3 and callus tissue (Figure 3C). The total IAA concentration in *Rhipsalidopsis* remains more or less constant during subculturing. The active *de novo* IAA synthesis and subsequent conjugation in *Schlumbergera* can be correlated with the auxin autotrophic growth reported by Sriskandarajah and Serek (2004).



**Figure 3.** Peroxidase (POX) activity (A), free IAA (B) and total IAA pool (free IAA + IAA-conjugates)(C) in greenhouse-grown leaves during two subsequent stages of *in vitro* subculturing and callus culture. Left (black bars), *Rhipsalidopsis* (cv. CB4); right (white bars), *Schlumbergera* (cv. Thor-Olga). All POX data are expressed as  $\mu\text{M} \text{mg protein}^{-1} \text{min}^{-1}$ , IAA and total IAA data are expressed as  $\text{pmol g}^{-1} \text{FW}$ .  $n = 3$ ; error bar: STERR; \*not analyzed.

### The Induced Peroxidase Activity during Subculturing Is Accompanied by an Increased Total ACC Pool in *Rhipsalidopsis*

*In vitro* grown *Rhipsalidopsis* tissue accumulates its total ACC pool during subculturing (Figure 4B). The endogenous concentration of free ACC increases during subculturing and is comparable between the two genera (Figure 4A). The enhanced total ACC pool may be caused by induction of ACC synthesis and concomitant ACC conjugation, where free ACC generally reflects ethylene biosynthesis.

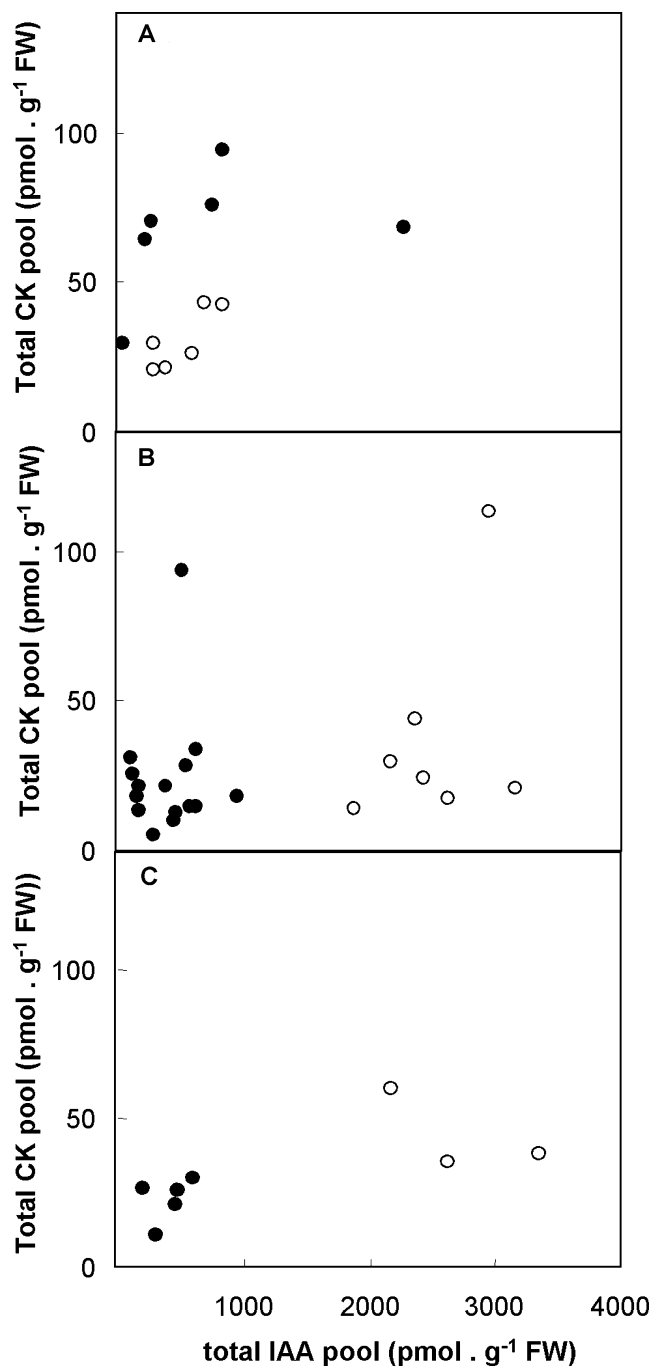


**Figure 4.** Free ACC (A) and total ACC pool (free ACC + ACC-conjugates) (B) in greenhouse-grown leaves during two subsequent stages of *in vitro* subculturing and callus culture. Left (black bars), *Rhipsalidopsis* (cv. CB4); right (white bars), *Schlumbergera* (cv. Thor-Olga). All data are expressed as  $\text{pmol g}^{-1} \text{FW}$ .  $n = 3$ ; error bar: STERR; \*not analyzed.

Ethylene biosynthesis, POX activity, and membrane breakdown appear to be closely linked (Arora and others 2002). Ethylene induces POX activity (Kato and others 2000) and stimulates degradation of IAA via oxidative decarboxylation (Winer and others 2000). In the absence of ethylene data, we cannot be conclusive about the causative factor in the interplay between POX activity, ethylene synthesis, and IAA homeostasis in *Rhipsalidopsis* and *Schlumbergera* tissue during subculturing.

### The Endogenous Auxin/Cytokinin Balance in *In vitro* Grown *Schlumbergera* Tissue Is Unfavorable for Adventitious Shoot Formation

The ratio between auxins and cytokinins is known to direct plant tissue toward adventitious root or adventitious shoot formation. The auxin/cytokinin ratio of each individual sample analyzed for both greenhouse leaves and *in vitro* tissues of *Rhipsalidopsis* and *Schlumbergera* was plotted (Figure 5) in a



**Figure 5.** CK-IAA balance in *Rhipsalidopsis* (cv. CB4) (closed symbols) and *Schlumbergera* (cv. Thor-Olga) (open symbols) projected on the Skoog and Miller diagram. Cytokinin and IAA levels were measured (expressed in  $\text{pmol} \cdot \text{g}^{-1} \text{FW}$ ) in initial explant samples (greenhouse-grown leaves) (A), in *in vitro* material originating from *in vitro* subcultures (B), and in calluses (C). All individual data are plotted.

classical Skoog and Miller diagram (Skoog and Miller 1957). High cytokinin levels favor adventi-

tious shoot formation and are unfavorable for root formation, whereas high auxin contents favor root formation but are unfavorable for adventitious shoot formation. The auxin/cytokinin ratio in greenhouse leaves of *Rhipsalidopsis* is slightly lower than the ratio in *Schlumbergera* (Figure 5A). In *in vitro* subcultures (Figure 5B) and in callus tissue (Figure 5C) of *Rhipsalidopsis*, the auxin/cytokinin balance remains constant, although there is a tendency toward lower levels of IAA and cytokinins. At the same time, a clear shift toward a high auxin/cytokinin ratio occurs during *in vitro* subculturing in *Schlumbergera* tissue (Figure 5B), and this shift is even more striking in callus (Figure 5C). However, this shift is considered to be unfavorable for adventitious shoot formation, and it appears to cause the recalcitrance of adventitious shoot formation observed for this genus.

Callus of *Rhipsalidopsis* is much more regenerative than that of *Schlumbergera* as reported earlier (Sriskandarajah and Serek 2004). This may be attributed to several characteristics such as high enzyme activities, actively proliferating cells, fewer anatomical barriers, and high uptake of hormones and nutrients. Liu and others (1997) demonstrated for soybean hypocotyl explants that the enhanced callus growth observed after exogenous application of naphthyl acetic acid and kinetin was accompanied by accumulation of endogenous IAA and polyamines. In the present study, callus appeared to have become more competent with age and addition of growth regulators, and therefore produced a large number of shoot primordia.

## GENERAL CONCLUSION

It is known that endogenous iP concentrations triggered the induction signal for an organogenetic response in pineapple leaf bases (Mercier and others 2003). This increase in iP levels required the exogenous supply of phytohormones such as the cytokinin BA and the synthetic auxin naphthyl acetic acid. Endogenous iP production was regulated by the uptake of these hormones, and at the same time induced CKX activity in the early stages of shoot development. Auer and others (1999) assigned the differences in organogenetic response of two petunia genotypes to differences in uptake and metabolism of exogenously applied BA, which subsequently affected the accumulation of endogenous isoprenoid cytokinins and the activity of CKX. Thus the treatment of *Rhipsalidopsis* and *Schlumbergera* explants by BA and IBA during *in vitro* subculturing in the experiments described here



explains the relatively high content of iP-type cytokinins in these tissues. Moreover, the CKX activity increased during subculturing. This increase was more pronounced in *Rhipsalidopsis* than in *Schlumbergera*, and it was negatively correlated with the total contents of isoprenoid cytokinins that serve as substrates for CKX. During subculturing of *Rhipsalidopsis*, a clear induction of POX occurred. This POX accumulation, in combination with auxin biosynthesis and conjugation rates, controls auxin homeostasis. The effect of subculturing on the CKX, and consequently, on cytokinin homeostasis appears less pronounced. POX versus CKX activity, in concert with an active auxin biosynthesis, could control the total auxin/cytokinin pool in favor of adventitious shoot formation in *Rhipsalidopsis*. The lack of accumulation of POX activity, in combination with auxin autotrophy, makes *Schlumbergera* more recalcitrant for adventitious shoot formation than *Rhipsalidopsis*.

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