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Alteration of Hormonal Levels in a Rootless Epiphytic Bromeliad in Different Phenological Phases

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Abstract. Major changes in indole-3-acetic acid (IAA) and cytokinin (CK) levels occur at different phenological phases of *Tillandsia recurvata* shoots. This epiphytic rootless bromeliad was chosen as suitable material for hormonal analysis because CK synthesis is restricted to the shoots, thus avoiding problems in the interpretation of results caused by translocation and interconversion of CK forms between roots and leaves encountered in plants with both organs. Young plants of *T. recurvata* have weak apical dominance because side shoots appeared early in development, and branch growth was correlated with a strong increase in the level of zeatin. The flowering phase was characterized by a significant increase in free base CKs, zeatin, and isopentenyladenine compared with the levels found in adult vegetative shoots. In contrast, both free-base CKs declined in the fruiting phenological phase, and the IAA level increased dramatically. It was concluded that in phases characterized by intense organ formation, such as in the juvenile and flowering stages, there was an enhancement of CK content, mainly caused by zeatin, leading to a lower IAA/CK ratio. Higher ratios were correlated with phases that showed no organogenesis, such as adult and fruiting phenologies.

Key Words. *Tillandsia recurvata*—Bromeliad—Auxins—Cytokinins—Plant development—Hormonal levels—Phenology

Tillandsia recurvata is an atmospheric epiphytic bromeliad called " ball moss" because of the stem's habit of curling around the host, thus giving a general ball-like appearance (Padila 1986) (Fig.1). This species is a member of the group known as rootless bromeliads. Nutrition and water uptake occur through foliar trichomes (Benzing 1990).

The developmental sequence of *T. recurvata* is illustrated in Fig. 2, and follows the typical pattern of growth and development in natural habitats. Seed germination occurs immediately after sowing. Young tillandsia plants have two leaf pairs that develop along the primary axis. A weak apical dominance is observed in this phase, and very soon in their lives side shoots, emerging from the primary axis, start to appear conferring a branching pattern. The mature growth form is characterized by the growth of side shoots, each consisting of four leaf pairs. In general, bromeliads of *Tillandsia* genus start flowering 5 to 8 years after the seedling phase (Isley 1987). The development of seed capsules is accompanied by a progressive decay of the parent plant, which dies slowly. When it is in decline, however, offshoots grow from one or more axillary leaf buds. Thus, the plant continues to be perpetuated by a sequence of axillary shoots.

Virtually all hormonal synthesis takes place in the shoots of *T. recurvata*, and the use of this bromeliad avoids interpretation problems associated with the possibility of translocation of cytokinins (CKs) between the roots and leaves encountered in plants that posses both organs.

Few studies have documented the changes in hormone levels throughout the life cycle of a plant (Slovin and Cohen 1988). However, a number of studies have attempted to correlate indole-3-acetic acid (IAA) and/or CK levels with particular developmental events, such as flowering (Kinet et al. 1993, Sotta et al. 1992,), senescence (Gan and Amasino 1996), and branching (Emery et al. 1998).

T. recurvata, a rootless plant, offers an excellent system for investigating hormone signals originating exclusively in the shoots and possibly involved in branch

Abbreviations: ABA, Abscisic acid—CK, Cytokinin—IAA, Indole-3-acetic acid—iP, Isopentenyladenine—iPA, Isopentenyladenosine—Z, Zeatin—ZR, Zeatin riboside.

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Fig. 1. Adult plants of *Tillandsia recurvata* in natural habitat.

development, flowering, and senescence. This exploratory study provides simultaneous measurements of IAA and four CKs, zeatin (Z), zeatin riboside (ZR), isopentenyladenine (iP), and isopentenyladenosine (iPA), using HPLC-ELISA analysis, of shoots of *T. recurvata* collected in the wild. Major changes in phytohormone levels were observed at defined phenological phases during the life cycle of this bromeliad.

Material and Methods

Plants

Tillandsia recurvata (Linnaeus) was found growing naturally on a host tree in the garden of the Department of Botany in the city of São Paulo, Brazil (Fig.1). Shoots were collected from this population at five phenological phases. Figure 2 shows the appearance of plants at each stage: in the germinating phase (Fig. 2*A*) the young plants with two pairs of leaves were about 1 year old; in the juvenile stage (Fig.2*B*), the plants show the branching pattern, each side shoot being about 1–2 cm long; in the adult phase (Fig.2*C*), the growth of the side shoots is at a maximum, each with four pairs of leaves; in the flowering phase (Fig. 2*D*), each side shoot develops a scape that bears one to three flowers (the inflorescence was not collected); in the fruiting phenology (Fig.2*E*), the shoots were collected 2 months after flower formation (fruit was eliminated for the analyses).

One gram of shoots from each phenological phase was immediately frozen in liquid nitrogen and stored at −20°C until extraction. Two replicates were measured for each phase. Each replicate consisted of 4–10 shoots.

Analysis of Hormones

The two groups of hormones were extracted and purified from the same sample. The immunoenzymatic method used for measuring the levels of IAA and CKs has been described in detail earlier by Sotta et al.(1987) and Pelèse et al. (1989). Four CKs were quantified: Z, ZR, iP, and iPA. Total CK content was considered to be the sum of these CKs.

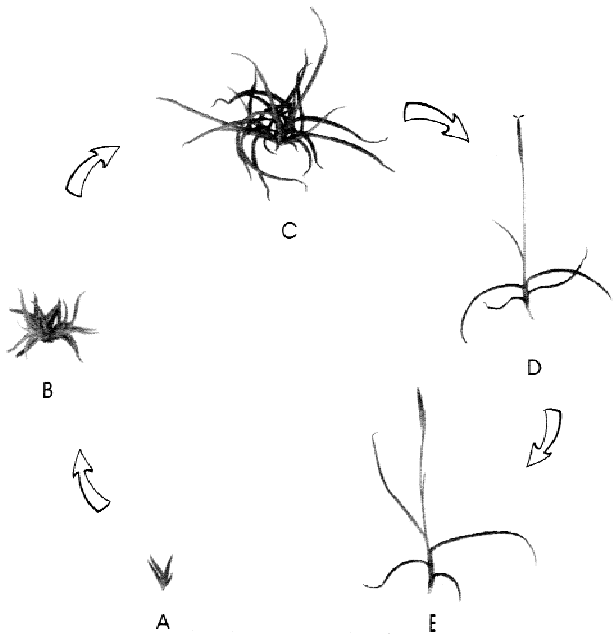

Fig. 2. Growth habits of *Tillandsia recurvata. (A)* Young plant with two leaf pairs. *(B)* Juvenile plant with four side shoots. *(C)* Adult plant showing four well developed branches. *(D)* Flowering branch of a plant. *(E)* Branch with seed capsule. In *D* and *E* the other branches of the whole plant were omitted.

Fresh samples (about 1 g) were homogenized in cold 80% methanol (v/v) containing 40 mg/L butylhydroxytoluene and stirred for 60 h at 4°C in this solvent. Tritiated standards (1.85 KBq of abscisic acid (ABA) and IAA per extract) were included in the samples for recovery estimation after purification. Tritiated IAA and ABA have been shown to perform as internal standards for Z, ZR and iP, iPA, respectively (Maldiney et al. 1986; Sotta et al. 1987). Extracts were filtered (0.45- and 0.2-μm mesh) and then passed through a Sep-Pack C_{18} cartridge eluted with 80% (v/v) methanol. The eluates were evaporated to the aqueous phase under vacuum and then diluted with 500 μL 0.2% v/v formic acid. HPLC was used for the separation of IAA and the four CKs from the prepurified extracts. Authentic IAA and CK standards were run with this system to establish their separation and relative retention time. The hormones were eluted from the reverse-phase HPLC column (Prep Nova-Pack HR C_{18}, Waters,) with a 0.2% formic acid (A)/ MeOH (B) gradient at a flow rate of 3 mL min^{-1}. For the gradient, the following protocol was used (%B in A in each case): 0–10 min, 18%; 10–11 min, 25%; 11–65 min, 33%; 65–70 min, 40%. The absorbance of the effluent was monitored at 265 nm. Fractions of 1.5-mL volume were collected and reduced to dryness in a Speed-Vac concentrator, after which samples were methylated with ethereal diazomethane for IAA analysis. The HPLC system was combined with ELISA because a relatively high content of UV-absorbing contaminants is present in the fractions. Thus the IAA and CKs could not be quantified from the records of UV monitor. The CKs were analyzed with polyclonal rabbit antibodies against iPA (60% cross-reactivity against iP) and ZR (90% cross-reactivity against Z) and the auxin with polyclonal rabbit anti-IAA antibodies. The level of each hormone per sample was measured four times and the results were collected for recovery. Calculations were made by reference to a calibration curve established on each microtitration plate with a fourth-order polynomial regression obtained from four experimental standard curves. All data presented here were obtained by means of analyses performed on one

set of experiments. However, the results of the second confirmed those of the first determination.

Results

IAA content

IAA level was initially low at germination and then increased almost fourfold at the juvenile phase. IAA concentration was higher in the flowering stage than in adult vegetative plants and reached a peak at fruiting (Fig.3).

Cytokinin Contents

At germination, total CK concentrations were low; as the young plants became older, at the juvenile stage, total CK concentrations increased strongly (Table 1) primarily because of an increase in Z (Fig. 3), which accounts for more than 80% of total CK concentrations at this stage. The juvenile stage contained the highest CK level measured in any of the *T. recurvata* phenological phases. Z and iP concentrations increased 68- and 72-fold, respectively, from germination to the juvenile phase, when the side shoots were about 1–2 cm long (Fig.3). Total CK levels decreased thereafter at the adult phase and then increased again when the flower emerged (Table 1). The levels of Z and iP were consistently higher in flowering plants than in nonflowering ones (sevenfold and eightfold higher, respectively) (Fig. 3). Z was predominant, amounting to 60% of total CK concentration at the flowering phase (Table 1). Total concentration of CKs declined considerably during the fruiting phenological phase, and the main type of CK found was the ribosylated form, ZR (53% of total CKs) (Table 1).

In all the phenological phases studied, iPA levels were extremely low (data not shown).

IAA/CK Balance

T. recurvata showed the highest IAA/CK ratio in the fruiting phase because of a decline in total CK concentration in association with a large increase in the IAA level (Table 1).

The germinating and adult phases also showed a high IAA/CK balance. However, for juvenile and flowering phenologies the opposite was observed. The lowest IAA/CK ratio was found at the juvenile stage and appeared consistent with the growing capabilities of side shoots.

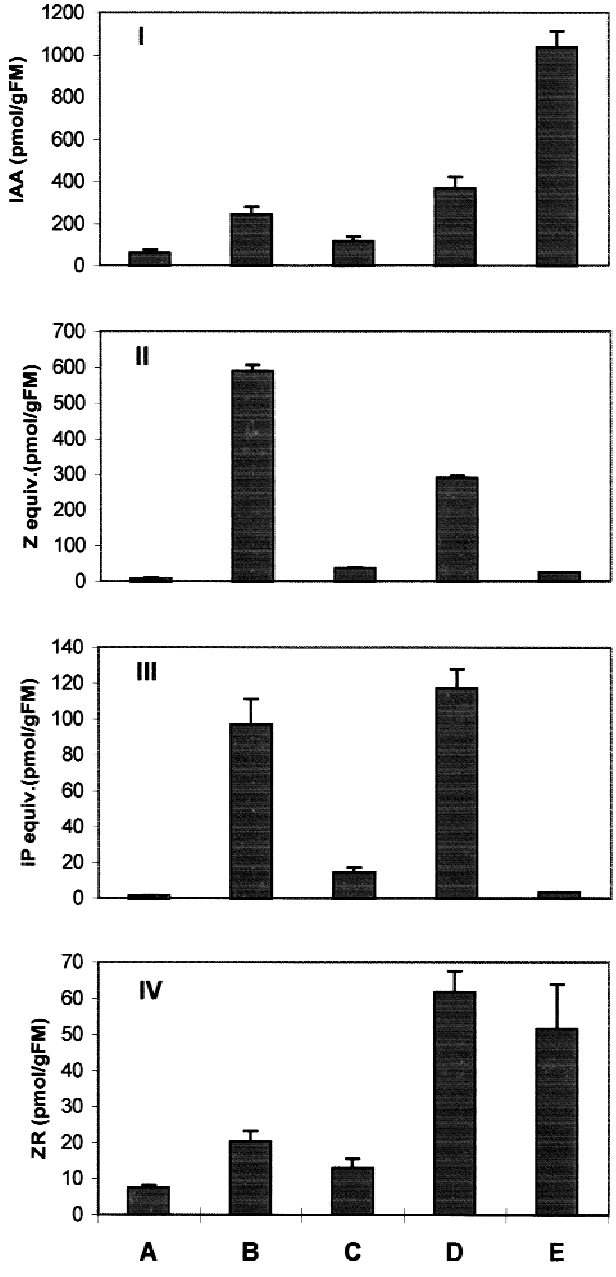

Fig. 3. Concentrations of IAA *(I)*, Z *(II)*, iP *(III)*, and ZR *(IV)* in shoots of *Tillandsia recurvata* at five phenological phases (*A*, germinating; *B*, juvenile; *C*, adult; *D*, flowering; *E*, fruiting). Data are mean ± SE (n = 4).

Discussion

Regulating the initiation of axillary meristems is an important mechanism for controlling overall plant form (Kerstetter and Hake 1997). In contrast to most bromeliads in which almost all axillary buds remain dormant until the shoot apex shifts from vegetative to reproductive growth, *T. recurvata* has a genetically determined developmental program that imparts the release of axillary buds from dormancy very soon in vegetative development. Thus young plantlets produce a branching stem system. Many workers believe that auxin from the shoot apex somehow influences the distribution and metabo-

Table 1. Total cytokinins, proportion of individual free base-, ribosylated-cytokinins, and auxin/cytokinin ratio in *Tillandsia recurvata* shoots at five phenological phases.

Phases	Total CKs	Z/ZR/iP/iPA	IAA/CKs
Germinating (A)	30.52 ± 4.6	0.28/0.25/0.04/0.43	2.02
Juvenile (B)	708.97 ± 21.2	0.83/0.02/0.12/0.03	0.34
Adult (C)	67.92 ± 2.8	0.55/0.2/0.21/0.04	1.75
Flowering (D)	473.07 ± 11.8	0.61/0.13/0.25/0.01	0.77
Fruiting (E)	97.02 ± 4.9	0.26/0.53/0.03/0.1	10.68

Data of total CKs are expressed as pmol g^{-1} FM ± SE.

lism of CKs from the roots and so promotes lateral bud outgrowth (Goodwin et al. 1978; Letham 1994; Woolley and Wareing 1972). Because *T. recurvata* has no functional root system, it is a good model to verify the interaction between CKs and auxin in controlling apical dominance at the shoot level. Bollmark et al. (1995) have proposed that polarly transported auxin may control bud outgrowth by regulating CK metabolic changes. Auxin might act by controlling the level of active CKs, for example by preventing iPA conversion to the more active zeatin (King and van Staden 1990). In the shoots of *T. recurvata*, we observed low total CK concentrations at the germinating phase, such that the IAA/CK ratio was high (Table 1). On the other hand, at the juvenile phase, total CKs increased strongly, caused mainly by a Z increase (83% of total). Our data support the hypothesis that the development of side shoots of this bromeliad is correlated with a marked synthesis of Z. Thus the decrease in the IAA/CK ratio may be part of an endogenous signal involved in the outgrowth of lateral shoots in *T. recurvata*.

The timing of the transition from vegetative growth to flowering is of great importance in agriculture, horticulture, and plant breeding (Bernier et al. 1993). Numerous studies to understand how this transition is controlled have indicated the important role of roots in the control of flowering. Root effects on flowering are commonly interpreted in terms of CK control (Vondráková et al. 1998). CKs are believed to represent one of the components in the multifactorial regulatory system of flowering (Bernier et al. 1993) and are synthesized predominantly in the roots (Palni et al. 1990), with subsequent transportation to the shoots where they can be interconverted to other types and then transported in the phloem to the apical bud (Lejeune et al. 1988, 1994). This is probably not the case for *T. recurvata* because this bromeliad has no functional root system, and most likely the shoots are capable of all CK synthesis. However, it was observed that total CK levels in the shoots of *T. recurvata* were significantly higher in flowering plants than in adult vegetative plants (Table 1). Significantly increased concentrations of mainly Z and iP were observed at the time of flower formation (Fig. 3), leading to a low IAA/CK ratio, despite a threefold increase in the IAA level. Floral transition in *Sinapsis alba* was characterized by a decrease of the auxin/CK ratio in the apical buds (Sotta et al. 1992). More recently, Chen et al. (1997) demonstrated that the promotion of flower bud development in *Euphoria longana*, an economically important fruit crop, was correlated with a dramatic increase in Z and ZR concentrations in the terminal buds. They also suggested that the promotion of floral differentiation might require a continuous supply of CKs from dormant buds and mature leaves. Dewitte et al. (1999) reported the CK distribution in tobacco shoot apices during floral transition and flower formation, showing that changes in the levels of endogenous CKs in the shoot apex itself seem to be involved in the flower transition. It was observed that the initiation of flower parts coincided with increased levels of the free bases (Z, DHZ, iP) and the corresponding ribosides. Flower formation was characterized by enhanced CK content in contrast to the low endogenous CK levels found in prefloral transition apices, which showed no organogenesis.

As the fruit develops in *T. recurvata*, it begins the sequential senescence of its somatic tissues. The progressive senescence in this bromeliad is accompanied by the release of some offshoots and culminates with the death of the mother-plant. The phenomenon of monocarpic senescence (plant death after reproduction) might be attributed to the vegetative meristems being starved of photosynthate because of diversion to the developing seed and fruit. This processes could be caused by hormone-directed transport or by seed and fruit acting as strong sinks (Wilson 1997). Hormones, especially CKs, can postpone monocarpic senescence (Neumann et al. 1983; Noodén et al. 1990). The shoots of *T. recurvata* in the fruiting phenological phase, although showing no visible signal of side shoot development, experience a strong decline in total CK concentration compared with the flowering phase (Table 1), mainly because of a marked decrease of Z and iP levels, whereas ZR concentration changed very little. In contrast, a large increase in IAA concentration was observed, contributing to the highest auxin/CK ratio found in any of the *T. recurvata* developmental phases. Hormonal levels could be thought of as coordinated signals, controlling the final phase of vegetative and reproductive development in *T. recurvata*, preceding the outgrowth of lateral shoots and death of the mother plant. The overall process of the senescence syndrome may be very complex, but there is no doubt that in certain contexts the timing of this conversion can be reversibly controlled by CK application (Bleecker and Patterson 1997). At the molecular level, CKs could inhibit senescence by suppressing the expression of senescence-associated genes and/or enhancing the activity of genes involved in photosynthesis and other cellular functions (Gan and Amasino 1996).

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