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BRIEF COMMUNICATION

Seasonal changes of cytokinins in upper and lower leaves of a sugar maple crown

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Abstract

Although it is well accepted that cytokinins (CKs) regulate processes such as leaf senescence and stomatal conductance, data on CKs in the canopy of mature trees are lacking in the literature. Here we report the first *in situ* sampling for determination of CKs in mature sugar maple (*Acer saccharum*) canopy layers. The upper canopy showed a distinct seasonal pattern in total CK content, while the lower canopy remained relatively unchanged.

Additional key words: *Acer saccharum*, canopy layers, liquid chromatography-tandem mass spectrometry.

What is known of hormone physiology in leaves of tree species comes almost exclusively from immature plants, with accounts of CK physiology being especially limited. The literature is also lacking in studies of the relationship between phytohormones and whole tree processes. A recent article by Atanasova *et al.* (2004) offered a rare glimpse into CK physiology in mature juniper (*Juniperus communis* L.) shoots. Nothing however, is known of seasonal trends in CK content of photosynthetically active tissues throughout a heterogeneous canopy. The present study addresses this deficiency directly by reporting the first seasonal profile of CKs in a mature deciduous tree species. We also explore the relationship between this profile and mature leaf physiology within the layers of a heterogeneous canopy. Microenvironental and tree-specific processes vary significantly from the top of the canopy to the forest floor. This ultimately creates a unique set of environmental pressures that influences the physiology of tissues found within each layer of the crown. The overall purpose of this study was to provide the first *in situ* study on the abundance and distribution of CKs in a mature deciduous species. We hypothesized that the physiological and climatic variation between the upper and lower canopy layers would result in altered CK accumulation.

Sample sites were selected in 4, individual mature (approx. 80 years old) trees of sugar maple (*Acer saccharum*) from the Trent University James MacLean Oliver Ecological Centre (44.57 °N, 78.5 °W). The lower canopy layer comprised foliage associated with the first level of branching at an average height of 2.2 ± 0.2 m, measured by lowering a graduated rope. The upper canopy layer comprised foliage in the upper crown of the tree canopy at an average height of 10.5 ± 1.0 m. A single cluster of foliage arising from the primary axis was selected and harvested at three times during the 2002 growing season to ensure representative sampling of the growth season. Sampling began as soon as the canopy was fully developed in 20 June and was repeated at 18 July, and late in 7 September. Instantaneous measurement of microclimatic variables including photosynthetically active radiation (PAR), relative humidity (RH) and temperature were collected during 11:00 - 14:00. Chlorophyll extraction followed protocols adapted

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Abbreviations: CK - cytokinin; PAR - photosynthetically active radiation; RH - relative humidity; DHZ - dihydrozeatin; [9R]DHZ - dihydrozeatin riboside; iP - isopentenyl-adenine; [9R]iP - isopentenyl-adenosine; Z - zeatin (*cis* or *trans*-Z); [9R]Z - zeatin riboside (*cis* or *trans*-[9R]Z).
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from Riemann (1980) and quantified according to Lichtenthaler (1987). Hormone extraction protocols for CKs were adapted from Emery *et al*. (1998). Eight independent, parallel samples were used to analyze each CK form. Leaf tissues (2 g per sample) were homogenized in cold (-20 °C) Bieleski's #2 (CH_3OH:H_2O:HCOOH [15:4:1, v/v]). During homogenization, 100 ng each of [$^2H_6$]iP, [$^2H_6$][9R]iP, [^{2}H]DHZ, [^{2}H][9R]DHZ, [^{2}H]-Z, and [^{2}H][9R]-Z, were added as internal CK standards. The pellet was then re-extracted twice in cold (-20 °C) Bieleski's #2. The combined supernatants were dried *in vacuo* at 38 °C. CKs were purified using *MCX SPE* columns following Dobrev and Kamínek (2002). CK free bases and ribosides were eluted using 5 cm^3 of 0.35 M NH_4OH in 60 % (v/v) MeOH. Elutes were dried *in vacuo* at 38 °C. Samples containing purified CKs were separated and analysed by liquid chromatography-tandem mass spectrometry (*LC/(+)ES-MS/MS*). The LC column was a *Genesis* C_{18} (150 × 2.1 mm) linked to a *Quattro II* (*Micromass*, UK) tandem mass spectrometer equipped with a positive electrospray interface. HPLC separation proceeded at a flow rate of 0.2 cm^3 min^{-1} with the following gradient: 8 - 15 % acetonitrile from 0 - 5 min and then to 100 % acetonitrile from 5 - 7 min. The mixing phase was 0.1 % formic acid in 20 mM ammonium acetate (pH 4.0). The detection limit for this instrument was approximately 2 pmol. All samples were quantified by multiple reactions monitoring of the mother and appropriate daughter ion as in Prinsen *et al*. (1995).

Seasonal variations in the leaf microclimate were observable between and within canopy layers (Table 1). PAR was found to be significantly different at all sampling dates between the upper and lower canopy layers. The PAR in the lower canopy decreased as the season progressed to a minimum of 28.13 µmol m^{-2} s^{-1}. In contrast, PAR increased in the upper canopy as the season progressed to a maximum of 448.81 µmol m^{-2} s^{-1}. PAR in the upper canopy was 4.5 - 16 times greater than in the lower canopy (*ANOVA*, $P \le 0.05$, $n = 16$) (Table 1). Relative humidity also displayed contrasting variation between canopy layers. In the upper canopy, relative humidity decreased in July, when compared to the increased humidity in the lower canopy (Student's *t*-test, $P \le 0.05$, $n = 16$). The temperature profile was not significantly different between canopy layers but there was significant variation within each layer (Table 1). No significant change in total chlorophyll content or the ratio of chlorophyll *a/b* was detected between or within the upper and lower canopy layers (data not shown).

Overall, the upper canopy displayed a more dynamic pattern of CK accumulation. Ribosides were found to accumulate in sugar maple crowns (Table 1), with [9R]iP being the most abundant CK form over all points. *Trans*-zeatin riboside (*trans*-[9R]Z) also accumulated in significant quantities in both canopy layers. For the upper canopy, the transition from June to July was associated with a significant increase in the content of [9R]iP and [9R]DHZ ($P \le 0.05$, $n = 16$) (Table 1), which contributed to an increase in total CK content observed between these two points to a maximum value of 89.00 pmol g^{-1}(f.m.) ($P \le 0.05$, $n = 16$) (Table 1). From July to September, the concentration of *cis*-[9R]Z, [9R]iP, and iP in the upper canopy decreased significantly ($P \le 0.05$, $n = 16$) (Table 1) and this translated into a significant decrease in total CK content to a minimum value of 45.43 pmol g^{-1}(f.m.) ($P \le 0.05$, $n = 16$) (Table 1). No significant trends in total CK were observable in the lower canopy ($P \le 0.05$)

Table 1. Seasonal variation in climatic conditions and hormonal content in upper and lower leaves of sugar maple crowns. CK measurements are reported in pmol g^{-1} (f.m.). Means ± SE, $n = 16$, * - values significantly different at $P < 0.05$ between lower and upper leaves, within a given month. A, B, C - values significantly different at $P < 0.05$ within each canopy layer, between two months, nd - not detected.

Parameter	Lower			Upper		
	June	July	September	June	July	September
PAR [µmol m^{-2} s^{-1}]	60.03 ± 5.20^A	43.91 ± 3.30^B	28.13 ± 1.72^C	258.09 ± 43.74*^{,A}	290.01 ± 41.88*^{,A}	448.81 ± 56.60*^{,B}
RH [%]	55.96 ± 0.79^A	60.70 ± 0.70^B	51.25 ± 2.22^A	48.41 ± 0.61^A	38.28 ± 0.58*^{,B}	60.76 ± 0.88^C
Temperature [^{o}C]	27.48 ± 0.20^A	26.46 ± 0.64^B	28.30 ± 0.68^A	26.93 ± 0.21^A	26.36 ± 0.22^A	25.64 ± 0.24^B
iP	16.50 ± 2.93^A	6.75 ± 0.62^B	8.63 ± 1.31^B	26.13 ± 7.30^A	7.63 ± 0.32^B	3.13 ± 0.40*^{,C}
[9R]iP	29.13 ± 3.92^A	43.86 ± 5.64^B	23.38 ± 1.84^A	28.50 ± 2.95^A	47.13 ± 3.57^B	21.00 ± 3.01^A
DHZ	nd	0.50 ± 1.41^A	4.86 ± 2.12^B	nd	nd	nd
[9R]DHZ	4.00 ± 0.96^A	6.75 ± 0.75^A	6.00 ± 0.73^A	5.00 ± 0.57^A	13.88 ± 1.84*^{,B}	11.63 ± 0.75*^{,B}
trans-[9R]Z	12.00 ± 1.82	17.29 ± 2.63	18.00 ± 2.25	15.67 ± 1.72	17.63 ± 2.03	18.33 ± 3.38
cis-[9R]Z	0.38 ± 0.74^A	1.50 ± 0.19^B	0.63 ± 0.35^A	1.13 ± 0.35^A	2.75 ± 1.19^A	0.17 ± 0.17^B
trans-Z	nd	nd	nd	nd	nd	nd
cis-Z	nd	nd	nd	nd	nd	nd
Total CK	62.50 ± 7.72	53.88 ± 7.62	60.25 ± 4.26	59.88 ± 6.16^A	89.00 ± 6.69*^{,B}	53.00 ± 7.09^A

(Table 1). The free-base dihydrozeatin (DHZ) was absent from the upper canopy at all sampling points, but accumulated in the lower canopy as the season progressed (Table 1). In addition to providing the first identification of CKs within mature *A. saccharum* trees, the present study provides a comparison of CK profiles and quantities between upper and lower canopies with respect to microclimatic variables. A clear seasonal trend of CK abundance was observed, particularly in the upper canopy. This profile was complemented by seasonal variation in microclimatic variables (PAR, RH), supporting the prediction that the physiological and climatic variations between the upper and lower canopy layers would result in altered CK content. [9R]iP was clearly the most abundant CK form at all sampling periods; it displayed a seasonal trend in both the upper and lower canopy layers of *A. saccharum*. The increased accumulation of [9R]iP in mid-season, upper canopy leaves was accompanied by the accumulation of other ribosides such as [9R]DHZ. By contrast, the lower canopy layer preferentially accumulated free-base forms (*i.e*. iP and DHZ) and ribosyl content remained low. These results imply that the specific accumulation of riboside and free-base forms is differentially regulated in leaves of contrasting canopy layers. Zeatin-type CKs are known to undergo *de novo* biosynthesis through either iPMP (isopentenyladenosine-5'-monophosphate) dependent (Miyawaki *et al.* 2004) or independent pathways (Astot *et al.* 2000). Once synthesised, the accumulation of active forms like [9R]DHZ in upper canopy leaves may be a result of the rapid metabolism of DHZ to [9R]DHZ, which has been noted in other species (Ahmadi and Baker 2000). It is also possible that the lower canopy leaves accumulates CK at a similar rate, but export the majority of these CKs to the upper canopy. Ribosyl CKs comprised the majority of accumulated forms during the September sampling period. Since ribosyl forms are believed to act as transport CKs (Emery and Atkins 2002), the accumulation of [9R]iP, [9R]DHZ, *cis*-[9R]Z and *trans*-[9R]Z in the upper canopy may be indicative of nutrient salvaging from tissues entering into senescence programs (Table 1). The microclimatic data collected provide a glimpse into environmental regulation of CK content. Classically, high chlorophyll *a/b* ratios have been attributed to sun-acclimated leaves, while low ratios are thought to be indicative of shade-acclimated leaves (Lambers *et al.* 1998). However, recent studies have presented conflicting data (Leal and Thomas 2003). Although light quality was significantly greater in the upper canopy, there was no apparent acclimation in leaves of the present study. Furthermore, a seasonal trend in CK abundance and profile in the upper canopy was observed that was not clearly related to chlorophyll *a/b* ratio, implying that CK accumulation may occur independently of this pathway. An intricate dissection of the complex relationship between CK content and changes in microclimatic variables is needed. In models of a simulated mixed *Quercus* - *Acer* forest canopy, it has been demonstrated that each canopy layer has an independent hydraulic system and that the upper layer, to avoid xylem cavitation, shows significant decreases in stomatal conductance (Williams *et al*. 1996). Currently, it is believed that stomatal closure is primarily controlled by ABA (Dodd 2003) and stomatal sensitivity to this hormone may vary in response to environmental conditions. Future experiments will compare CK and ABA concentrations complemented by direct measurements of stomatal aperture and density. The data of the present study indicate that the accumulation of CKs is differently regulated in leaves of the upper and lower canopy layers. The accumulation of specific forms may also fluctuate within each canopy layer throughout the growing season, which may be related to changes in the microclimate of the leaf.

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