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Full Length Research Paper

Endogenous cytokinin levels during early fruit development of macadamia

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Cytokinins play a central role in fruit set and development, with many plants accumulating high concentrations of endogenous cytokinins in developing fruits, and applied cytokinins being used to induce fruit set or parthenocarpic fruit development. Cytokinin application to flowers or immature fruit of macadamia increases initial fruit set and delays fruit abscission, without affecting final fruit set. This study quantified and determined the timing of endogenous cytokinin accumulation in macadamia flowers and immature fruit, to assist in developing a cytokinin treatment to improve fruit set and kernel quality. Flowers and very young fruit contained little cytokinin but levels rose greatly between 3 and 10 weeks after anthesis. Zeatin and zeatin riboside were the predominant cytokinins at 10 weeks post-anthesis, with zeatin riboside concentrations being 20 - 230 times higher than concentrations detected immediately after anthesis. Cytokinin accumulation coincides with the period of maximum endosperm volume in macadamia which, along with the predominance of zeatin and zeatin riboside, conforms to the general pattern of accumulation in developing fruits of many other plants. The very high cytokinin concentrations at this stage may explain the inability of pre- or post-anthesis cytokinin applications to increase fruit retention of macadamia beyond 10 weeks post-anthesis.

Key words: Benzyladenine, benzylaminopurine, fruit drop, kernel, nut, plant growth regulators, Proteaceae.

INTRODUCTION

Macadamia (*Macadamia integrifolia*, *M. tetraphylla* and hybrids) is a mass-flowering tree, indigenous to the subtropical rainforests of eastern Australia, and grown extensively in commercial plantations in South Africa, Australia, Hawaii and Brazil. Macadamia flowers are produced in spring on racemes bearing 100 - 300 flowers (Trueman and Turnbull, 1994a; Wilkie et al., 2009) but more than 98% of the flowers and immature fruits abscise during the first 8 - 10 weeks after anthesis (Sakai and Nagao, 1985; Trueman and Turnbull, 1994b; Trueman, companion paper). Mature fruits abscise and are harvested from the ground more than 14 weeks later (Nagao and Sakai, 1988; Trueman et al., 2000, 2002; Trueman 2003a, b) to provide the nut-in-shell and edible kernel (Walton and Wallace, 2008, 2009). Several plant growth regulators have been tested for their ability to reduce abscission of macadamia flowers and immature fruits but only the cytokinin, benzyladenine (BA), has shown any promise of increasing fruit retention (Williams, 1980; Trueman, 2010).

Cytokinins are often considered to play a central role in fruit set and development (Rock and Quatrano, 1995; Morris, 1997; Ozga and Reinecke, 2003). Developing fruits of many plants contain high concentrations of endogenous cytokinins, usually during early stages of fruit development (Hernandez and Primo-Millo, 1990; Brenner and Cheikh, 1995; Lewis et al., 1996a; Arnau et al., 1999; Emery et al., 2000; Tarkowski et al., 2006; Boonkorkaew et al., 2008), and cytokinins are used to induce fruit set or parthenocarpic fruit development when applied exogenously (Lewis et al., 1996b; Vivian-Smith and Koltunow, 1999; Yu et al., 2001; Maroto et al., 2005; Watanabe et al., 2008; Zhang et al., 2008). In macadamia, BA application to flowers prior to anthesis or to fruits 3 weeks post-anthesis can delay abscission for up to 6 weeks, but these BA treatments do not increase fruit retention beyond 10 weeks post-anthesis (Trueman, 2010). Cytokinins have been identified and quantified in sap, apical and lateral buds of macadamia (Fletcher and Mader, 2007), but there have been no reports on endogenous cytokinins in macadamia fruits. Such information is useful for developing an exogenous cytokinin treatment to improve macadamia yield and kernel quality. This paper describes the identification and

Table 1. Abbreviations used for cytokinins.

ZR5P	Zeatin riboside 5´-monophosphate
dHZR5P	Dihydrozeatin riboside 5´-monophosphate
dHZ9G	Dihydrozeatin-9-glucoside
iPA5P	N^6-isopentenyladenosine 5´-monophosphate
Z	Zeatin
ZR	Zeatin riboside
dHZ	Dihydrozeatin
dHZR	Dihydrozeatin riboside
iP9G	N^6-isopentenyladenine-9-glucoside
iPA	N^6-isopentenyladenosine
iP	N^6-isopentenyladenine

quantification of endogenous cytokinins in developing macadamia fruits, and discusses their relationship with the abscission-delaying effects of exogenous cytokinins (Trueman, 2010).

MATERIALS AND METHODS

Fruit samples

Fruits were collected from macadamia cultivars 'H2' ('Hinde') and '246' ('Keauhou') at Hidden Valley Plantations, Beerwah, Queensland (26°50'S 152°56'E) and from cultivar '660' ('Keaau') in an orchard managed by Macadamia Plantations of Australia Pty Ltd at Wollongbar, New South Wales (28°49'S 153°23'E). Racemes were selected and tagged on four trees of each cultivar, approximately 3-days pre-anthesis. Each sample consisted of all the fruits from four racemes, with one raceme from each tree. Fresh weights of all samples were recorded, and samples were stored in liquid nitrogen.

All selected racemes of cvv. H2 and 246 were pollinated using the test-tube method described by Trueman and Turnbull (1994a). Cultivar H2 racemes were pollinated using cv. 246 pollen, and cv. 246 racemes were pollinated using cv. H2 pollen. Samples of these cultivars were collected 7, 14, 21, 30, 36, 42, 56 and 70 days post-anthesis. Flowers of cv. 660 were not hand-pollinated. Samples of this cultivar were collected 3, 21, 42, 56, 70, 87, 101 and 115 days post-anthesis. Extractions were performed on entire samples except for the 56 – 70-day (cvv. H2 and 246) and 42 – 115-day (cv. 660) samples, where only one or two fruits were used per extract due to the higher fresh weights of individual fruits. Samples up to 7-day post-anthesis included sepals that had not abscised from some flowers.

Extraction procedure

Tissue samples were homogenised with an Ultra-Turrax tissue disintegrator together with 90% methanol (10 mL g^{-1} fresh weight) and 330 Bq of isopentenyl adenosine-[^{3}H]-diol (2.18×10^{11} Bq $mmol^{-1}$). Extracts were cleared by centrifugation. Insoluble material was reextracted in 90% methanol. The combined extracts were reduced to an aqueous residue by rotary evaporation at 35°C. The extract was frozen to -20°C, then thawed and centrifuged (10000 g for 5 min) to remove precipitated lipids and chlorophyll. The extract was then passed through a column of insoluble polyvinylpolypyrrolidone (4 mL g^{-1} fresh weight) to remove phenolic compounds. Five volumes of water were then passed through the column to recover the cytokinins. The eluate was rotary evaporated to < 3 ml.

When the sample fresh weight was > 3 g, the eluate was adjusted to pH 8.0 with NaOH or KOH, and extracted 4 times with 3 ml of water-saturated n-butanol. The combined n-butanol fractions were rotary evaporated to dryness and then dissolved in 3 ml water.

The extract was then passed through a SepPak C_{18} cartridge (Waters). The cartridge was washed with 6 mL water, and cytokinins were recovered by eluting with 6 ml of 60% methanol. This fraction was rotary evaporated to dryness.

HPLC

Cytokinins were fractionated on a Shandon ODS 5 µm Hypersil column (250 × 4.6 mm) using a gradient of methanol in water at a flow rate of 1 mL min^{-1}. The water phase was adjusted to pH 6.8 with 5 mM triethylammonium acetate. All dissolved samples were centrifuged at 10000 g for 5 min to remove particulate matter prior to injection. Samples were fractionated using one of two different solvent gradients.

Most 4 - 14 day post-anthesis samples from cvv. H2 and 246 were fractionated using the following system. Samples were dissolved in 900 µL of 45% methanol. Two sequential solvent programs were employed - (a) 45% methanol for 15 min: iPA-type cytokinins were collected in 15 × 0.5 min fractions from 6 min, all other cytokinins being recovered in a single fraction from 1 - 6 min which was rotary evaporated, redissolved in 900 µL of 22% methanol, and reinjected, with a mobile phase of (b) 22% methanol with 22 × 0.5 min fractions collected from 2 - 13 min.

All other samples were fractionated using the following system. Samples were dissolved in 900 µL of 20% methanol. The methanol gradient used was: 20 – 30% over 1 min; 30% for 9 min; 30 - 50% over 1 min; 50% for 9 min; 50 - 100% over 1 min; 100% for 5 min. From 2 - 24.5 min, 45 x 0.5 min fractions were collected.

All collected fractions were dried in a Savant SVC100H centrifugal sample concentrator at 45°C. Fractions were then dissolved in water. 200 µL was used for the 15 iPA-type fractions from the first solvent system and fractions 30 - 45 from the second solvent system, 300 µL was used for the 22 ZR and dHZR fractions from the first solvent system, and 350 µL was used for fractions 1 - 29 from the second solvent system (abbreviations for cytokinins are listed in Table 1).

Recoveries of isopentenyl adenosine-[^{3}H]-diol internal standards were estimated from the appropriate HPLC fractions to be 3 - 72%. Low isopentenyl adenosine-[^{3}H]-diol recoveries were associated with samples of high fresh weight (> 3 g) which yielded > 10000 pg of detected ZR in the 50 µL of sample assayed (that is, levels greater than the upper limit of detection).

Radioimmunoassay

Radioimmunoassay was performed on all HPLC fractions. Cytokinins were measured using three different antisera, raised against ZR, dHZR and iPA. Each antiserum had high cross-reactivity with all cytokinins (base, nucleoside, nucleotide and 9'-glucoside) possessing the same N^6 side-chain, but low cross-reactivity (< 5%) with other cytokinins.

All samples and standards were assayed in duplicate. Each assay tube contained 100 µL Tris-HCl buffer (0.1 M pH 7.1) plus 0.1 M NaCl, 200 µg bovine immunoglobulin and 170 Bq tracer (zeatin ribosyl-[^{3}H]-diol, dihydrozeatin ribosyl-[^{3}H]-diol or isopentenyl adenosyl-[^{3}H]-diol; cytokinin tracers had specific radioactivities of 1.7 - 2.2×10^{11} Bq $mmol^{-1}$, corresponding to a [^{3}H]-cytokinin concentration of 4 - 5 nM). To this was added 50 µL of standard (ZR, 50 - 10000 pg; or dHZR, 50 - 10000 pg; or iPA, 50 - 20000 pg)

Table 2. Cytokinin concentrations (pmol g^{-1} fresh weight) in macadamia cv. H2 fruits.

	Time post-anthesis (days)								
	2	**4**	**11**	**14**	**21**	**30**	**42**	**56**	**70**
ZR5P	nd	nd	3	nd	nd	nd	3	nd	nd
dHZR5P	7	26	1	11	5	6	4	1	3
dHZ9G	5	10	6	4	7	nd	8	nd	2
iPA5P	2	2	1	2	2	nd	nd	nd	nd
Z	nd	nd	37	nd	27	190	212	357	1322
ZR	11	37	4	25	66	242	467	786	2553
dHZ	nd	150	34	46	nd	nd	222	104	80
dHZR	5	5	1	4	6	7	9	5	23
iP9G	nd	nd	nd	nd	nd	nd	nd	nd	nd
iPA	16	16	5	6	7	10	24	19	40
iP	nd	10	16	nd	30	39	17	5	4

'nd' = not detected. Mean fresh weights of individual fruits from 2 – 70 days post-anthesis were 0.0040, 0.0028, 0.0101, 0.0063, 0.0123, 0.09, 0.36, 4.02 and 8.66 g, respectively.

Table 3. Cytokinin concentrations (pmoles g^{-1} fresh weight) in macadamia cv. 246 fruits.

	Time post-anthesis (days)										
	2	**4**	**7**	**11**	**14**	**21**	**30**	**36**	**42**	**56**	**70**
ZR5P	nd	8	8	6	nd	nd	nd	8	7	nd	nd
dHZR5P	7	6	9	13	8	47	34	38	12	2	2
dHZ9G	4	2	30	11	10	nd	10	12	6	2	2
iPA5P	nd	nd	2	3	nd	3	4	6	nd	nd	nd
Z	nd	nd	43	134	61	nd	125	586	1177	578	1005
ZR	20	nd	nd	nd	11	73	192	377	1630	777	1179
dHZ	86	nd	56	48	78	nd	nd	581	181	70	94
dHZR	6	1	4	3	nd	9	24	26	10	12	12
iP9G	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
iPA	14	12	8	9	7	15	30	15	30	37	90
iP	nd	16	11	31	9	32	96	114	7	13	12

'nd' = not detected. Mean fresh weights of individual fruits from 2 – 70 days post-anthesis were 0.0027, 0.0029, 0.0041, 0.0037, 0.0055, 0.0138, 0.07, 0.14, 0.69, 3.15 and 9.65 g, respectively.

or sample. Water was used for determination of B_{max}. Finally 50 µL of appropriate diluted antiserum was added (water for non-specific binding bank) and the tubes were mixed and incubated for 1 h. Next, 200 µL of saturated ammonium sulphate solution was added, thoroughly mixed and left to precipitate the antibody-antigen complexes for 20 min. Tubes were centrifuged (10000 g for 3 min) and supernatants discarded. Pellets were washed with 150 µL of 50% saturated ammonium sulphate, centrifuged as before and decanted again. Pellets were dissolved in 225 µL water, mixed thoroughly for 1 h, before 1.2 mL of Beckman ReadyValue scintillation cocktail was added. Tubes were counted on a Packard Minaxi 4000 scintillation counter for 2 min. Data was processed using a custom-written program. The assays were calibrated using a least-squares linear regression plot of logit $[B/B_{max}]$ v. log [standard concentration]. Compounds were identified on the basis of immunoreactivity and HPLC retention times, compared with standard cytokinins.

Where ZR levels beyond the standard curve were detected (that is, > 10000 pg), fractions were re-assayed after dilution (1:5 for cvv. H2 and 246; 1:10 and 1:20 for cv. 660). Extract dilution curves were parallel to the standard curves, and results from the 1:5 and 1:10 dilutions are reported. The lowest standard (50 pg) was regarded as the detection limit for all samples.

RESULTS AND DISCUSSION

Compounds with HPLC and radioimmunoassay behaviour of Z, ZR, ZR5P, dHZR, dHZR5P, dHZ9G and iPA (Table 1) were detected in extracts of all three macadamia cultivars, H2, 246 and 660 (Tables 2, 3 and 4 respectively). In addition, dHZ, iP and iPA5P were

Table 4. Cytokinin concentrations (pmol g^{-1} fresh weight) in macadamia cv. 660 fruits.

	Time post-anthesis (days)							
	3	**21**	**42**	**56**	**70**	**87**	**101**	**115**
ZR5P	nd	5	nd	nd	nd	nd	nd	nd
dHZR5P	11	12	47	15	4	nd	nd	nd
dHZ9G	12	nd	16	8	6	nd	nd	nd
iPA5P	nd	nd	nd	nd	nd	nd	nd	nd
Z	nd	nd	nd	nd	157	nd	nd	nd
ZR	25	10	100	87	547	329	66	64
dHZ	nd	nd	nd	nd	nd	nd	nd	nd
dHZR	15	45	6	12	15	15	3	3
iP9G	nd	15	2	nd	nd	nd	nd	nd
iPA	83	13	6	146	18	8	5	9
iP	nd	nd	nd	nd	nd	nd	nd	nd

'nd' = not detected. Mean fresh weights of individual fruits from 3 - 115 days post-anthesis were 0.0156, 0.0170, 0.76, 1.24, 3.24, 9.98, 11.61 and 16.49 g, respectively.

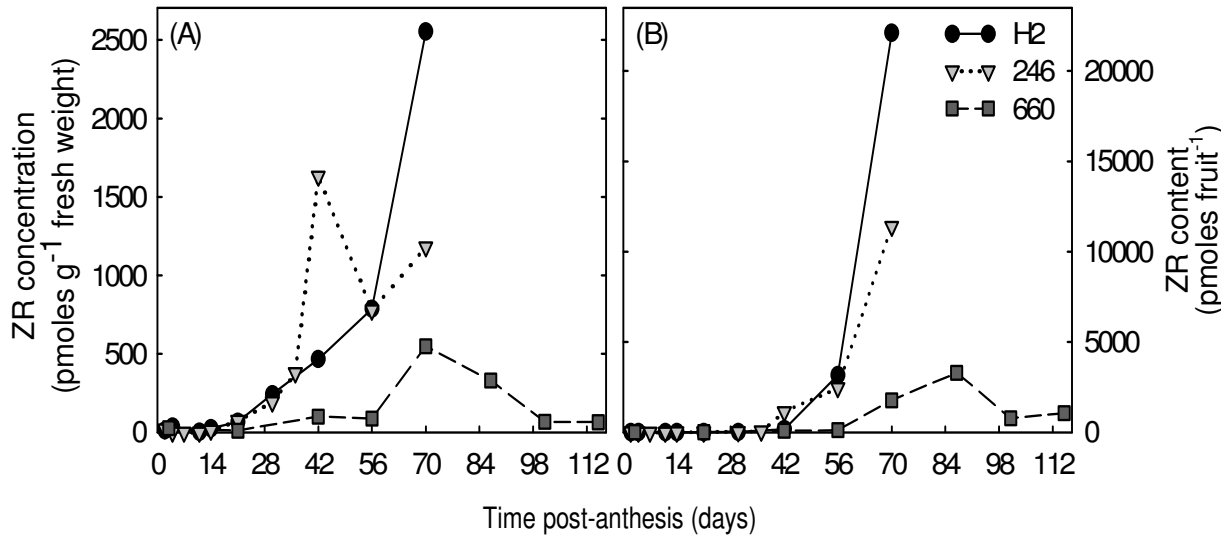

Figure 1. Extractable zeatin riboside concentrations (A) and total contents (B) in the whole fruits of macadamia cultivars, H2, 246 and 660.

detected in extracts of cvv. H2 and 246, and iP9G were detected in extracts of cv. 660. The low levels and inconsistent presence, though, of several cytokinins (particularly ZR5P, iPA5P and iP9G) places uncertainly on the validity of their detection. Several of the cytokinins detected in macadamia fruit (viz. Z, ZR, dHZR, iPA and iP) have been previously identified in sap, apical or lateral buds of macadamia cv. 344 (Fletcher and Mader, 2007).

The levels of extractable cytokinins rose rapidly beyond 21 days post-anthesis, with very high levels present at 70 days post-anthesis for all three cultivars. At this stage, ZR (Figure 1A and B) and Z were the predominant cytokinins, but Z was not detected at any other stage in cv. 660 (Table 4). ZR concentrations for cvv. H2, 246 and 660, respectively, were 2553, 1179 and 547 pmoles g^{-1} fresh weight at 70 days post-anthesis, compared with 11, 20 and 25 pmoles g^{-1} fresh weight at 2 or 3 days post-anthesis. These figures represent approximately 230-, 60- and 20-fold increases in ZR concentrations over this period; they also represent approximately 500,000-, 200,000-, and 4,000-fold increases in total ZR content per fruit. Where extractable cytokinin levels were determined beyond 70 days post-anthesis (in cv. 660), a decline was observed between 87 and 101 days post-anthesis. ZR concentrations fell from 329 to 66 pmoles g^{-1} fresh weight, an approximately 8-fold decline from the

concentration observed at 70 days post-anthesis (that is, 547 pmol g^{-1} fresh weight). Because of the very rapid increase and subsequent decline in cytokinin levels observed around this time, it is possible that maximum cytokinin levels were even higher than those evident from fortnightly sampling. The concentrations of individual cytokinins were much higher at this stage than those detected previously in xylem sap and buds of macadamia; that is, from <1 to 33 pmol g^{-1} fresh weight (Fletcher and Mader, 2007).

The free nuclear endosperm of macadamia fruit develops rapidly for several weeks before cell walls are laid down around 56 days (Hartung and Storey, 1939) or 70 – 77 days post-anthesis (Strohschen, 1986). Embryo development is more gradual, but the cotyledons have completely replaced the cellular endosperm by 140 days post-anthesis (Hartung and Storey, 1939; Strohschen, 1986). The highest cytokinin levels in developing macadamia fruits are, therefore, observed around the time of maximum endosperm volume, following low cytokinin levels around anthesis. These features, along with the apparent predominance of Z and ZR, conform to the general patterns of cytokinin accumulation in developing fruits of many other plants (Brenner and Cheikh, 1995; Morris, 1997; Ruffini, 1998; Arnau et al., 1999; Emery et al., 2000; Tarkowski et al., 2006; Liu et al., 2010).

The very high endogenous cytokinin concentrations observed around 70 days post-anthesis may explain, at least partly, the inability of pre- and post-anthesis cytokinin applications to increase macadamia fruit sets at or beyond this stage, despite increasing fruit sets up to 56 days post-anthesis (Trueman, 2010). Cytokinin levels were low during the first 21 days post-anthesis, but macadamia fruits are highly sensitive to applied cytokinins at this stage (Trueman, 2010). Abscission of immature macadamia fruit occurs in three distinct phases, with maxima at 2, 6 - 7 and 10 weeks post-anthesis (Trueman and Turnbull, 1994b). Pre-anthesis BA applications increase fruit sets after the first phase by 2.7- to 6.2-fold and, in the case of repeated applications, after the second phase by 1.3- to 2.2-fold, but they do not affect retention of fruits beyond the third phase (Trueman, 2010).

Macadamia fruits may, in effect, be cytokinin-saturated and insensitive to exogenous cytokinins around 70 days post-anthesis. In addition, however, BA may have delayed abscission of fruits that were destined to abscise (Peterson et al., 1990; Kuang et al., 1991; Atkins and Pigeaire, 1993). Fruit retention during the second and third phases is strongly related to available carbohydrate levels, with these phases possibly representing a maternal adjustment of crop load prior to the major period of fruit biomass accumulation (Trueman and Turnbull, 1994b; Trueman and Wallace, 1999). Fruit retention after 56 days post-anthesis may become resource-limited, with the extra macadamia fruits induced by exogenous cytokinin simply being shed during a subsequent period of crop load adjustment.

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