Endogenous gibberellin levels during early fruit development of macadamia

Stephen J. Trueman

Faculty of Science, Health and Education, University of the Sunshine Coast, Maroochydore DC 4558, Australia.
E-mail: strueman@usc.edu.au. Tel: +61 7 54565033.

Gibberellins play a key role in flower and fruit development, and fruits often contain high gibberellin concentrations. This study quantified endogenous gibberellins in flowers and immature fruits of the macadamia cultivars, H2 and 246, to assist in developing growth-regulator treatments for improving fruit set. GA$_1$, GA$_3$, GA$_8$, GA$_9$ and GA$_{20}$ was detected in flowers or fruits of both cultivars, and GA$_4$ were detected in flowers of cultivar 246. The timing of gibberellin accumulation was consistent between cultivars but total concentrations were very low (< 7 pmol g$^{-1}$ fresh weight). Low gibberellin levels are consistent with the inability of the gibberellin-synthesis inhibitor, paclobutrazol, to influence fruit retention in macadamia.

Key words: Abscission, fruit drop, kernel, nut, paclobutrazol, plant growth regulators, Proteaceae.

INTRODUCTION

Macadamia (Macadamia integrifolia, Macadamia tetraphylla and hybrids) is a subtropical evergreen tree that is grown in plantations in South Africa, Australia, Hawaii and Brazil. Macadamia trees produce masses of racemes in spring, each bearing 100 to 300 flowers (Trueman and Turnbull, 1994a; Olesen et al., 2011). More than 98% of flowers and immature fruits abscise during the first 10 weeks after anthesis (Trueman and Turnbull, 1994b). Fruits reach maturity about 24 weeks post-anthesis, after which they fall to the orchard floor and are harvested and dehusked to provide the nut-in-shell and edible kernel (Trueman et al., 2000, 2002; Walton and Wallace, 2008, 2009).

Auxins, cytokinins, gibberellins and the gibberellin-synthesis inhibitor, paclobutrazol, have been tested on macadamia to reduce flower and fruit drop but none has increased final fruit set (Williams, 1980; Trueman, 2010a). The cytokinin, benzyladenine, increases fruit retention for up to 8 weeks after anthesis but does not increase fruit set beyond the major abscission period at 10 weeks post-anthesis (Trueman, 2010a). Endogenous cytokinin levels are very high in macadamia fruits around 10 weeks post-anthesis, which suggests that fruits may be, in effect, cytokinin-saturated and insensitive to applied cytokinins at this stage (Trueman, 2010b).

Gibberellins also play a key role in flower and fruit development (Wilkie et al., 2008; de Jong et al., 2009), and fruits often contain high levels of endogenous gibberellins (Zhang et al., 2007; Ayele et al., 2010). This paper describes the identification and quantification of gibberellins in developing macadamia fruits, and discusses their relationship with the effects of gibberellic acid and paclobutrazol applications on fruit retention (Trueman, 2010a). Such information is useful for developing growth regulator treatments to improve macadamia yield and kernel quality.

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). Racemes were selected and tagged on four trees of each cultivar, approximately 3 d pre-anthesis (28 Sep 1989). All selected racemes were pollinated using the test-tube method of Trueman and Turnbull (1994a) and Wallace et al. (1996), with cv. H2 racemes pollinated using cv. 246 pollen, and cv. 246 racemes pollinated using cv. H2 pollen. Fruit samples were collected 2, 4, 7, 11, 14, 21, 30, 36, 42, 56 and 70 days post-anthesis. Each sample consisted of all the fruits from four racemes, with one raceme from each tree. Samples at 2 to 7 days post-anthesis also included sepals that had not yet abscised from some fruits. Fresh weights of all samples were recorded, and samples
were stored in liquid nitrogen. Extractions were performed on entire samples except for the 56 to 70 days samples, where only two fruits were used per extract due to the higher fresh weights of individual fruits.

**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 [³H]GA₃ (1.21 x 10¹⁴ 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 40°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 (6 ml g⁻¹ fresh weight) to remove phenolic compounds. Three volumes of water were then passed through the column to recover the gibberellins. The eluate was rotary evaporated to < 3 ml, adjusted to pH 2.9 with HCl, and extracted 5 times with 2/3 volume of ethyl acetate. The combined ethyl acetate fractions were washed with water to remove excess acid, rotary evaporated to dryness, dissolved in 2 ml methanol, and then desiccated. The gibberellins were methylated with ethereal diazomethane, and the sample was again rotary evaporated to dryness.

**HPLC**

Gibberellins were fractionated on a Shandon ODS 5 µm Hypersil column (250 x 4.6 mm) using a gradient of methanol in water at a flow rate of 1 ml min⁻¹. Samples were firstly dissolved in 900 µl of 30% methanol and centrifuged at 10000 g for 5 min to remove particulate matter prior to injection. Samples were then fractionated using the following gradient: 35% for 4 min, 35 to 47% over 7 min, 47 to 87% over 10 min, 87 to 100% over 5 min, and 100% for 5 min. Thirty-five 0.6-min fractions were collected from 6 to 27 min. All collected fractions were dried in a Savant SAVC100H centrifugal sample concentrator at 45°C, and then dissolved in 200 µl water. Recoveries of [³H]GA₃ were estimated from the appropriate HPLC fractions to be 6 to 73%.

**Radioimmunoassay**

Radioimmunoassay was performed on all HPLC fractions. An antiserum against GA₃ was used. This antiserum had a broad specificity for C₃-GAs, which allowed accurate simultaneous measurement of GA₁, GA₃, GA₄, GA₅ and, at lower sensitivity, GA₆ and GA₉. 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 ([³H]GA₃-Me, corresponding to a concentration of 0.7 nM). To this was added 50 µl of standard (GA₃-Me, 5 – 5000 pg) or sample. Water was used for determination of Bmax. Finally 50 µl of appropriate diluted antiserum was added (water for non-specific binding blank) 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 three standards and a least-squares linear regression plot of logit [B/Bmax] v. log [standard concentration]. Compounds were identified on the basis of immunoreactivity and HPLC retention times, compared with standard gibberellins. The lowest standard (5 pg) was regarded as the detection limit for all samples.

**RESULTS**

Compounds with HPLC and radioimmunoassay behaviour of GA₁, GA₃, GA₅, GA₆ and GA₂₀ were detected in extracts of both cultivars, H2 (Table 1) and 246 (Table 2). GA₆ was only detected in the 2- and 4-days post-pollination extracts of cv. 246 flowers. The timing of gibberellin accumulation was consistent between cultivars (Figure 1), but the total concentrations of extractable gibberellins were very low (max. 6.31 pmol g⁻¹ fresh weight) throughout the first 70 d after anthesis (Tables 1 and 2).

**DISCUSSION**

Macadamia fruits contained several gibberellins (viz. GA₁, GA₃, GA₅, GA₆ and GA₂₀) that have also been detected at low concentrations (max. 11.48 pmol g⁻¹ fresh weight) in apical buds or lateral buds of macadamia cv. 344 (Fletcher and Mader, 2007). The gibberellin levels in macadamia fruits (< 7 pmol g⁻¹ fresh weight) were much lower than those of other species such as walnut (Carya species) and pistachio (Pistacia vera) (Mader, 2006). Gibberellin levels in macadamia fruits were also lower than those detected in other nut crops. This may be due to the fact that macadamia fruits have a longer maturation period than the other crops. Future studies should focus on the role of gibberellins in the development and ripening of macadamia fruits.
Table 2. Gibberellin concentrations (pmol g\(^{-1}\) fresh weight) in macadamia cv. 246 fruits.

<table>
<thead>
<tr>
<th></th>
<th>2</th>
<th>4</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>30</th>
<th>36</th>
<th>42</th>
<th>56</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA(_1)</td>
<td>nd</td>
<td>0.37</td>
<td>nd</td>
<td>0.83</td>
<td>1.09</td>
<td>0.40</td>
<td>0.55</td>
<td>0.55</td>
<td>0.46</td>
<td>0.40</td>
</tr>
<tr>
<td>GA(_3)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.87</td>
<td>0.55</td>
<td>1.24</td>
<td>nd</td>
<td>nd</td>
<td>1.71</td>
<td>2.57</td>
</tr>
<tr>
<td>GA(_4)</td>
<td>0.36</td>
<td>0.51</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>GA(_8)</td>
<td>0.47</td>
<td>0.74</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.80</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>GA(_9)</td>
<td>0.44</td>
<td>nd</td>
<td>0.47</td>
<td>nd</td>
<td>1.11</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.32</td>
</tr>
<tr>
<td>GA(_{20})</td>
<td>1.11</td>
<td>0.99</td>
<td>nd</td>
<td>0.75</td>
<td>1.84</td>
<td>nd</td>
<td>0.45</td>
<td>0.48</td>
<td>1.33</td>
<td>0.87</td>
</tr>
<tr>
<td>Total</td>
<td>2.38</td>
<td>2.61</td>
<td>0.47</td>
<td>2.45</td>
<td>5.39</td>
<td>1.64</td>
<td>1.00</td>
<td>1.03</td>
<td>3.50</td>
<td>4.16</td>
</tr>
</tbody>
</table>

'nd' = Not detected.

Figure 1. Fresh weight of individual fruit (A) and total contents of extractable gibberellins in the fruit (B) of macadamia cultivars, H2 and 246.

lower than the levels detected, for example, in early developing fruits of tomato and pear (~ 250 and 662 pmol g\(^{-1}\) fresh weight, respectively) and in almost-ripe fruits of Christmas rose (~ 483 pmol g\(^{-1}\) fresh weight) (Serrani et al., 2007; Zhang et al., 2007; Ayele et al., 2010). Macadamia buds do, however, contain higher levels of GA\(_7\) (Fletcher and Mader, 2007). GA\(_4\), GA\(_7\) and GA\(_{20}\) are most likely converted from GA\(_6\), and the other gibberellins are then produced directly (GA\(_1\)) or indirectly (GA\(_3\) and GA\(_8\)) from GA\(_{20}\) (Sponsel, 1995; Ward et al., 2010).

The current results show that macadamia flowers and fruits contain very low gibberellin levels during the periods of fertilization, endosperm development and early embryo development. Macadamia pollen tubes reach the ovary within 7 days of pollination (Trueman and Turnbull, 1994a; Wallace et al., 1996) and endosperm develops rapidly for several weeks before cell walls are laid down between 35 and 77 days post-anthesis (Hartung and Storey, 1939; Sedgley, 1981; Strohschen, 1986). Embryo development is more gradual, but the first zygotic division occurs approximately 35 days post-anthesis and the endosperm is completely replaced by cotyledons by 140 d post-anthesis (Hartung and Storey, 1939; Sedgley, 1981; Strohschen, 1986). The very low gibberellin concentrations during endosperm and early embryo development of macadamia contrast with many other species, where gibberellin levels are high during early fruit development (Serrani et al., 2007; Zhang et al., 2007; Ozga et al., 2009; Ward et al., 2010).

The very low gibberellin levels in macadamia flowers and immature fruits explain, in part, the inability of paclobutrazol applications to influence fruit retention during the early phases of flower and fruit drop (Trueman, 2010a). Paclobutrazol inhibits the conversion of ent-kaurene to ent-kaurenol early in the gibberellin synthesis pathway and, thus, reduces endogenous gibberellin levels (Sponsel, 1995). Immature macadamia fruits contain very low gibberellin levels and are, therefore,
unlikely to respond to gibberellin-synthesis inhibitors. Fruit drop is also unaffected by GA₃ application (Trueman, 2010a), which tends to indicate that the processes involved in macadamia fruit retention are not sensitive to gibberellin concentrations.

Abscission of immature macadamia fruits occurs in three phases around 2, 6 and 10 weeks post-anthesis (Trueman and Turnbull, 1994b). The first phase results from fertilization failure in some flowers, but the second and third phases are strongly related to available carbohydrate levels, possibly representing an adjustment of crop load prior to the major period of biomass accumulation (Trueman and Turnbull, 1994a, b; Trueman and Wallace, 1999; McFadyen et al., 2011). Future attempts to regulate macadamia yield through the use of plant growth regulators could focus on treatments that manipulate resource supply and source-sink relationships during the final stages of immature fruit drop around 10 weeks post-anthesis.

ACKNOWLEDGEMENTS

Thanks to Henry, Alison and David Bell (Hidden Valley Plantations) for orchard access and assistance, Colin Turnbull, Bill Slater and Helen Wallace for advice, assistance and critical reading of earlier versions of the manuscript, Kerry Anderson and Elektra Grant for technical assistance, and the Australian Macadamia Society, CSIRO, and the Horticultural Research and Development Corporation for financial support.

REFERENCES


