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Delay of early fruitlet abscission by branch girdling in citrus coincides with previous increases in carbohydrate and gibberellin concentrations

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Abstract Current evidence in citrus indicates that gibberellins (GAs) are main determinants of early fruit set while subsequent growth of developing fruits is mostly dependent upon carbohydrate availability. In this work, branch girdling performed at anthesis in Satsuma mandarin (*Citrus unshiu* (Mak.) Marc.) cv. Okitsu transitorily reduced early abscission rates (12–32 days after anthesis, DAA) delaying initially the process of natural fruitlet drop. The effects of girdling on growth, gibberellin (GA) and carbohydrate concentrations in developing ovaries and fruitlets were assessed during this initial growth stage (0–69 DAA). In girdled branches, abscission rate reduction was preceded by elevated concentrations of carbohydrate and GA in developing ovaries and fruitlets. Girdling at anthesis stimulated higher hexose (21 DAA) and starch (6–20 DAA) concentrations and also higher GA_1 (6 DAA), GA_{19} (13–20 DAA) and GA_{20} (6–20 DAA). The results established a relationship between the reduction of early abscission rates and higher concentrations of carbohydrates and GAs induced by girdling in developing fruitlets. These findings revealed that girdling certainly increased GA concentration and strongly suggested that its effect on early fruitlet abscission delay is likely mediated by both GA and carbohydrates.

Keywords Gibberellins · Hexoses · Okitsu satsuma · Starch · Sucrose

Abbreviations

cv	Cultivar
DAA	Days after anthesis
DW	Dry weight
GAs	Gibberellins

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Introduction

It is generally accepted that in citrus the number of growing fruits that survive after June drop is mainly determined by nutritional factors such as photoassimilate (Mehouachi et al. 1995; Gómez-Cadenas et al. 2000). Thus, a strong relationship between carbohydrate availability for fruitlet growth and the likelihood of abscission has been suggested (Iglesias et al. 2003). This suggestion has also been described for other tree species is supported by studies on translocation of ^{14}C metabolites, fruit abscission and manipulation of fruit to leaf ratio (Atkinson et al. 2002; Blanusa et al. 2006; Downton et al. 1987). Moreover, leaf photosynthesis appears to be crucial in determining fruit set (Iglesias et al. 2002) and decreases in leaf number (Mehouachi et al. 1995) or inhibition of photosynthetic activity (Beruter and Droz 1991) have been reported to induce abscission. On the contrary, treatments like girdling resulted in low fruit abscission rates, through the increase of carbon availability for the fruitlets above the girdle (Iglesias et al. 2003). Therefore, there is good evidence that sugars are involved in the regulation of fruit abscission during the June drop.

There is also evidence, with citrus, showing a strong hormonal component regulates ovary and fruitlet abscission. Thus, gibberellins (GAs) are thought to be pivotal effectors responsible of the transition of ovary to fruit (Talon et al. 1992; Ben-Cheikh et al. 1997). GAs are activators of cell division and cell enlargement processes, and their presence is generally associated with the initiation of both phases of growth. The endogenous GAs found in citrus fruits are mainly members of the 13-hydroxylation pathway (GA_{53}, GA_{97}, GA_{44}, GA_{17}, GA_{19}, GA_{20}, GA_{29}, GA_1, epi-GA_1, and GA_8; Talon et al. 1990a, b, 1992) leading to GA_1, the bioactive GA. This notion is supported by several studies reporting that exogenous GA_3 considerably improves fruit set of self-incompatible genotypes with negligible parthenocarpic fruit set (Soost and Burnett 1961). These genotypes also contain lower GA_1 concentrations than the seedless varieties that show natural parthenocarpy (Talon et al. 1992). In developing ovaries, GA_1 concentrations are low before and after anthesis and rise just at anthesis. This transitory increase in GA_1 concentrations can be detected in parental seeded genotypes as well as in seedless mutants possessing high or normal ability for fruit set (Talon et al. 1990a, b).

The above information suggests that the balances of sugars and GAs are involved in the set and abscission during the cell division phase of citrus fruit growth.

On the other hand, it is known that girdling produces many growth alterations including a decline in fruit abscission. The effects of bark girdling have been suggested to be due to the transitory blocking of phloem transport from leaves to roots that may increase availability of sugars and hormones above the girdle (Goldschmidt et al. 1985; Wallerstein et al. 1978; Yamanishi 1995). The enhancement of carbohydrate availability has been associated with an improvement of fruit set and yield of citrus trees (Monselise et al. 1972; Goldschmidt 1999; Goren et al. 2003). It has been also reported that girdling improves citrus fruit set through the increase of leaf photosynthetic capacity (Iglesias et al. 2006; Rivas et al. 2007). Together, it is suggested that in addition to the control of June fruit drop, sugars may also be implicated in the regulation of ovary and fruitlet abscission that occurs shortly after anthesis. The involvement of GAs in citrus fruit set in girdled trees has been scarcely investigated; however, it was reported that girdling reduced total GA-like activity in young fruitlets of Shamouti orange (Wallerstein et al. 1973).

The objective of this work was to elucidate, in citrus, the involvement of sugars and GAs on the early abscission of developing ovaries and fruitlets in response to girdling.

Materials and methods

Plant material

Mandarin trees of Satsuma mandarin cv. Okitsu (*Citrus unshiu*, (Mak) Marc) grafted on Carrizo citrange (*Citrus sinensis*, Osb. × *Poncirus trifoliata*, Raf) rootstocks were used in these experiments. Okitsu cv. of Satsuma mandarin shows moderate dwarfism (1.5–2 m tall) and was used previously in several citrus fruit abscission studies (Mehouachi et al. 1995, 2000; Gómez-Cadenas et al. 2000). Plants were 7-year-old and grown in experimental fields at the Instituto Valenciano de Investigaciones Agrarias (Moncada, Valencia, Spain).

Treatments and growth measurements

Girdling was performed on small trees (1.5 m height) at anthesis and consisted of a circular cut (1 mm width) around the bark of the basis of secondary or tertiary branches (6–10 cm diameter). In a parallel experiment, 200 fruitlets distributed over at least 4 branches per tree were treated at anthesis with GA_3 (1 µg per fruitlet). GA_3 (20 µg/ml) was dissolved in

5% (v/v) aqueous ethanol containing 0.05% (v/v) Tween 20 and then applied by dipping fruitlet in the solution for 5 s, which delivered about 50 µl of solution per fruitlet. Control fruitlets were treated with water containing ethanol and Tween 20.

Growth measurements were carried out regularly from anthesis to the end of the cell division phase (69 DAA approximately). Developing ovaries and fruitlets, in general, 30 g of fresh weight were sampled periodically during this interval. After growth measurements, samples were lyophilized, ground and stored at −20°C until analyses. Fruit drop was recorded on populations of 200 tagged fruitlets from anthesis to 69 DAA.

Gibberellin extraction and analysis

The method followed for gibberellin (GA) quantitation was previously described in detail (Ben-Cheikh et al. 1997; Gutierrez-Mañero et al. 2001). In brief, determination of GAs involved sequential purification through adsorption, polyvinylpolypyrrolidone and ion exchange chromatography. Additional purification was performed through C_{18} Sep-pack cartridges and reverse-phase HPLC. Fractions of HPLC containing the GA of interest were derivatized and separately analysed using a Fisons 8000 gas chromatograph coupled to a quadrupole MD 800 Fisons mass spectrometer. The samples (2 µl) were injected in splitless, the helium (He) inlet pressure was 85 KPa and the injector, interface and MS source temperatures were 250, 250 and 200°C, respectively. Ion electron impact masses at 70 eV were acquired in selected ion monitoring (SIM) mode. For quantification, different amounts of internal standards were used and ions were monitored (dwell times = 80 ms) as follows: for GA_{19}/[$^2H_2$]GA_{19}-MeTMSi, *m/z* 436 and 434; GA_{20}/[$^2H_2$]GA_{20}-MeTMSi, *m/z* 420 and 418 and GA_1/[$^2H_2$]GA_1-MeTMSi, *m/z* 508 and 506.

Carbohydrate analysis

The procedure for carbohydrate analysis was previously described (Mehouachi et al. 1995; Iglesias et al. 2003). In brief, 100 mg of powdered samples were extracted with 1.0 ml 80% ethanol and purified sequentially by cation and anion exchange columns. The eluates were then passed through a C_{18} Sep-pak cartridge (Waters-Millipore, Barcelone, Spain) and analysed using a Waters HPLC system (Waters-Millipore, Mildford, MA) equipped with a WISP 710B autosampler processor, a 510 pump, a high performance carbohydrate column (4.6 × 250 mm, Waters-Millipore) and a R401 differential refractometer. Sucrose, glucose and fructose were identified by their retention times.

Statistical analyses and reproducibility of the determinations

Four independent plots, of five trees each, per treatment (control and girdling) were established for fruit growth and abscission, carbohydrate and GA determinations. Fruit growth and abscission measurements were recorded from anthesis to 69 DAA and data were the average of 50 and 200 fruitlets, respectively, in each plot. Abscission rates were calculated as the percentage of dropped fruitlets each period relative to the number present at the beginning of that period.

One independent determination of carbohydrate and GAs in each plot was performed in fruitlets harvested following a random sampling from anthesis until either 20 (starch and GAs) or 69 DAA (fructose, glucose, sucrose). For each determination, at least two extractions from the same sample were carried out and similar concentrations were obtained.

For GA quantification, different amounts of GA_{19}/[17-$^2H_2$]GA_{19} (95.9% enrichment), [17-$^2H_2$]GA_{20} (99.6% enrichment) and [17-$^2H_2$]GA_1 (99.6%) were added to the methanolic extracts as internal standards. For carbohydrate quantification, known amounts of fucose were added to the ethanolic extracts as internal standards (Mehouachi et al. 1995).

Data were generally compared using the least significant difference (LSD) test ($P \leq 0.05$). Statistical analyses were performed using Statgraphics Plus for Windows, V.2.1 (Statistical Graphics Corp., MD, USA).

Results

Fruit growth and abscission

Changes in fruit dry weight (DW) of Okitsu cv. showed the typical pattern for citrus fruit growth. Fruit DW increased progressively during the cell

Table 1 Fruit dry weight of cv. Okitsu in control plants and plants subjected to branch girdling at anthesis

Treatments	Fruit dry weight (g)	
	Control	Girdling
DAA		
0	0.012 ± 0.001a	0.012 ± 0.001a
12	0.042 ± 0.008a	0.045 ± 0.011a
21	0.071 ± 0.016a	0.076 ± 0.014a
32	0.250 ± 0.058a	0.307 ± 0.094a
41	0.724 ± 0.014a	0.636 ± 0.006a
54	1.618 ± 0.057a	1.636 ± 0.017a
69	3.236 ± 0.011a	3.126 ± 0.057a

Values of four blocks were compared for each date ($n \geq 50$ per block) using the least significant difference (LSD) test. The data presented in each column followed by the *same letter* do not differ significantly at ($P \leq 0.05$). *DAA* days after anthesis

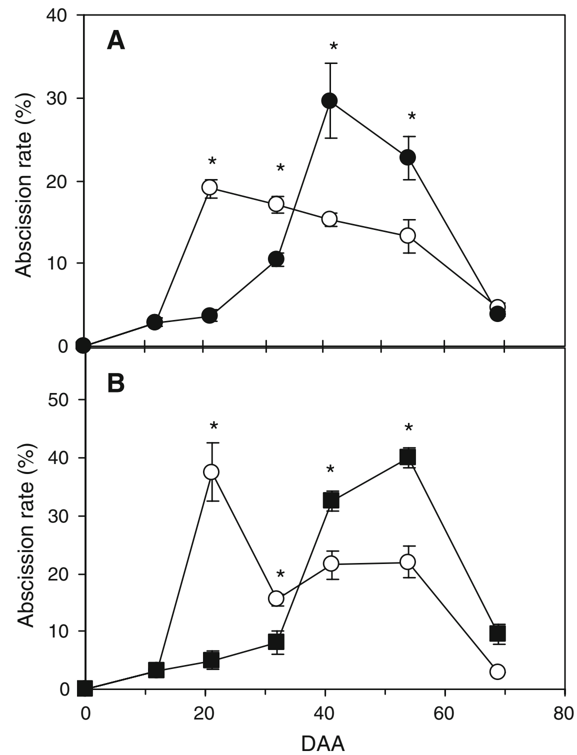


Fig. 1 Effects of branch girdling (**a**) and GA_3 treatment (**b**) on fruitlet abscission rate (%) in trees of Okitsu cv. (○), control; (●), girdling; (■),GA_3 treatment. Girdling and GA_3 application were performed at anthesis and data are the means ± SE of 200 fruitlets per block, with four blocks per treatment. Significant differences at $P \leq 0.05$ are represented by an *asterisk* (*). *DAA* days after anthesis

division stage until the end of the study, 69 days after anthesis (DAA). Control fruits reached at this date, DW values close to 3.2 g while girdling carried out at anthesis did not alter either the final DW or the pattern of fruit growth (Table 1).

Fruit abscission was assessed from anthesis until 69 DAA (Fig. 1a, b). In control trees, fruitlet abscission rate reached a maximum of 20%, 21 DAA, and thereafter it decreased continuously. However, in girdled trees, abscission rate decreased during the first 32 DAA in comparison to controls, and then increased to reach a maximum (30%), 41 DAA. Subsequently, fruitlet abscission showed a similar decrease in both control and girdled trees, resulting in the same rates of abscission (Fig. 1a). In a parallel experiment, fruitlet abscission rate was evaluated in trees subjected to exogenous treatments with GA_3 at anthesis (Fig. 1b). As above, GA_3 reduced abscission rate relative to the control trees at 21 and 32 DAA; however, afterwards, abscission rate of GA_3-treated fruits increased in comparison to control ones. At the end of the experiment (69 DAA) abscission rate declined in control and treated fruits to reach low values.

Carbohydrate changes

Soluble sugars were analysed in developing fruitlets of control and girdled trees from anthesis to 69 DAA. In control plants, fructose varied from 3.4 to 4.4 mg g^{-1} DW during the first 32 DAA and subsequently decreased drastically to show its lowest concentration (about 1 mg g^{-1} DW, 41 DAA). Thereafter, fructose accumulated to reach 6.3 mg g^{-1} DW at the end of the experiment (Fig. 2a). In girdled trees, fructose changes along fruit growth were similar to those recorded in control fruits except for one single date. Thus, fruits on girdled branches at 21 DAA, contained higher fructose concentrations (40%) than fruits on control branches. Similarly, glucose concentration in control fruitlets as in normal citrus fruit growth conditions was initially low, reached its lowest values 41 DAA (4 mg g^{-1} DW) and increased thereafter. Again, fruits from girdled trees showed the same pattern of changes in glucose compared to control fruits except for a significant increase of glucose concentration at 21 DAA (Fig. 2b). Sucrose concentrations in fruitlets

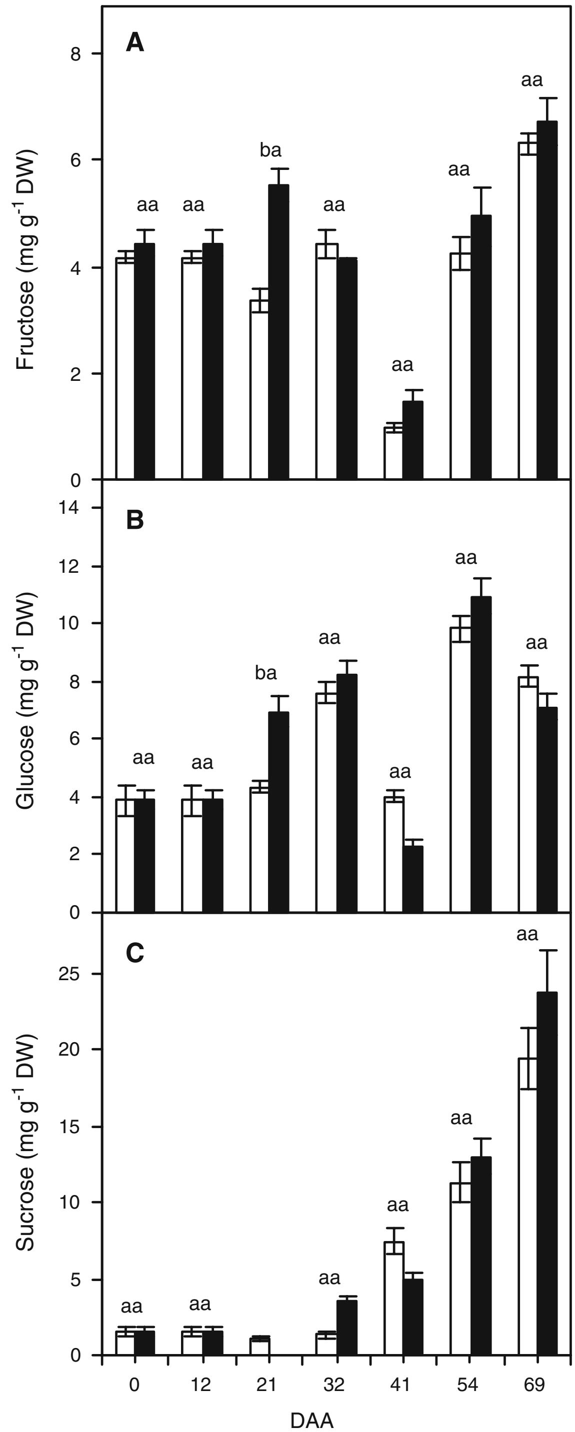


Fig. 2 Fructose (**a**), glucose (**b**) and sucrose (**c**) levels in developing fruitlets of Okitsu cv. in control plants (□) and plants subjected to branch girdling (■). Girdling was performed at anthesis and data were compared for each date ($n \geq 4$) using the least significant difference (LSD) test. *Dissimilar letters* above columns differ significantly at $P \leq 0.05$. *Error bars* show SE. *DAA* days after anthesis

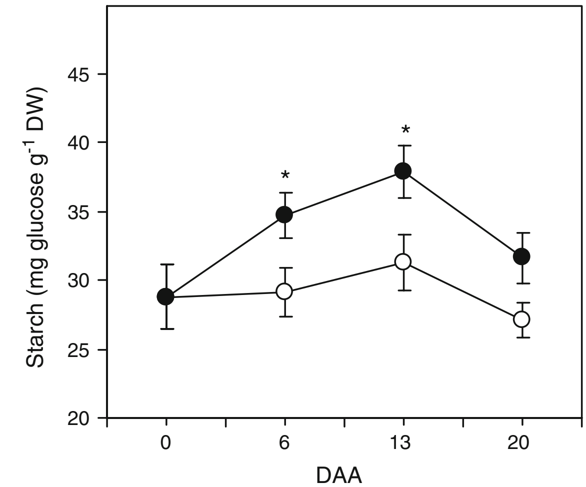


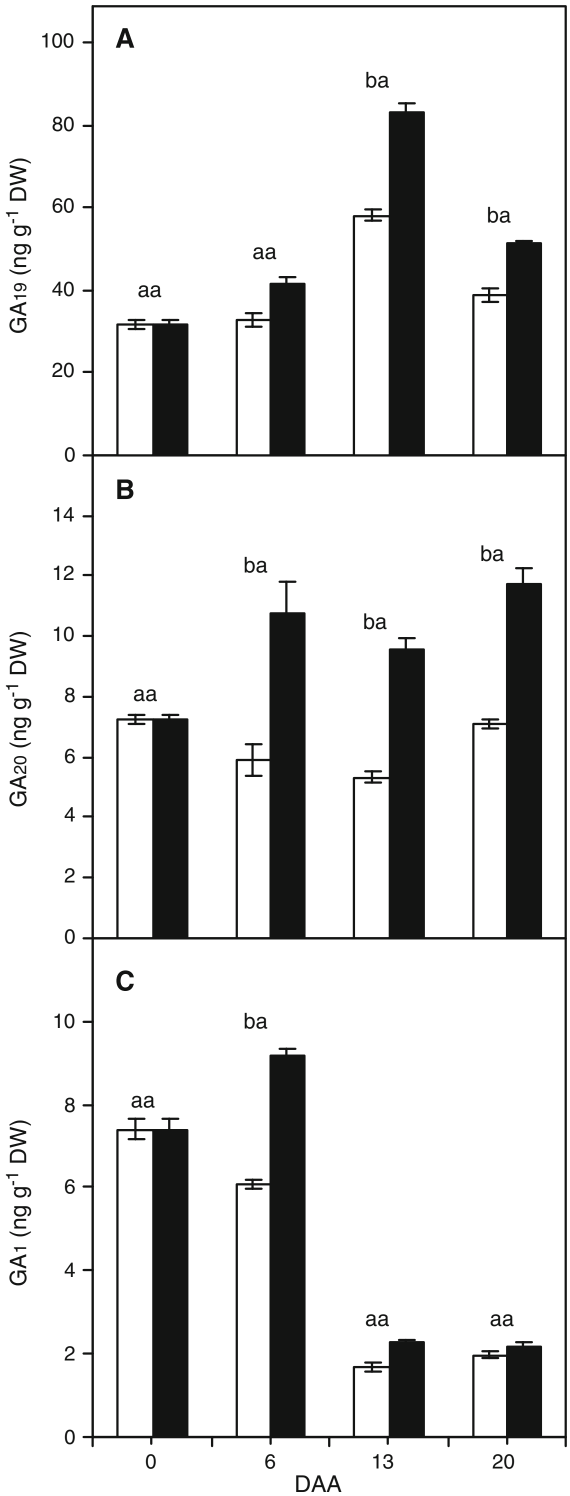
Fig. 3 Starch levels in developing fruitlets of Okitsu cv. in control plants (○) and plants subjected to branch girdling (●). Girdling was performed at anthesis and values are means ± SE of four independent determinations. Significant differences at $P \leq 0.05$ are represented by an *asterisk* (*). *DAA* days after anthesis

were also low during the initial phases of growth (0–32 DAA) and increased continuously afterwards (Fig. 2c). Girdling did not modify the fruit sucrose accumulation pattern.

Starch concentration in control fruits was fairly constant (Fig. 3), while girdling significantly increased starch in developing fruitlets over 0–13 DAA period, and maintained this concentration higher than control at 20 DAA. The highest differences were reached 13 DAA (17%).

Gibberellin changes

Gibberellin determinations were carried out in fruitlets from anthesis until 20 DAA. The analyses were focused on the active GA in citrus, GA_1, and its immediate precursors GA_{20} and GA_{19} (Fig. 4a–c). In control fruitlets, GA_{19} concentrations oscillated between 32 and 58 ng g^{-1} DW, showing a peak 13 DAA. Girdling applied at anthesis increased GA_{19} accumulation relative to the control at 13 DAA (30%) and at 20 DAA. Concentration of GA_{20} in control fruitlets ranged from 7.3 to 5.3 ng g^{-1} DW, along the studied period. In girdled fruits GA_{20} increased in all samples (39–45%) in comparison to control. The concentration of GA_1 in control fruits diminished progressively from anthesis to 20 DAA



◀ **Fig. 4** Endogenous gibberellin levels (GA_{19} (**a**), GA_{20} (**b**) and GA_1 (**c**)) in developing fruitlets of Okitsu cv. in control plants (□) and plants subjected to branch girdling (■). Girdling was performed at anthesis and data were compared for each date ($n \geq 4$) using the least significant difference (LSD) test. Dissimilar letters above columns differ significantly at $P \leq 0.05$. *Error bars* show SE. *DAA* days after anthesis

(Fig. 4c). Thus, the highest concentrations were found at anthesis (7.4 ng g^{-1} DW) and decreased thereafter to reach the lowest values 13 and 20 DAA (1.7 and 2 ng g^{-1} DW, respectively). Shortly after girdling, GA_1 concentration strongly increased (34%, 6 DAA) relative to control although there were no statistical differences at 13 and 20 DAA.

Discussion

The data presented here showed that branch girdling performed at anthesis in Okitsu mandarins significantly reduced early fruitlet abscission rates (12–32 DAA), delaying temporarily natural fruitlet drop. Afterwards, abscission rates in treated fruits increased and as a result, cumulative abscission was similar in girdled and non-girdled fruit (Fig. 1a). It is interesting to note that the pattern of abscission rates observed in girdled trees was similar to that produced by a single application of exogenous GA_3 (Fig. 1b), suggesting that one of the main effects of the hormone was to delay abscission. Taken together, these observations established a link in developing fruitlets between a reduction in abscission rates and increased concentrations of GAs and carbohydrates in girdled trees. GAs appear to be required for fruitlet abscission decrease, while carbohydrates seem to be necessary for fruit development. Data suggest that sugars and GAs were key determinants of fruit set in citrus. This suggestion agrees compelling evidence generated in this field during several years. Here we show that the positive effect of girdling on delaying early abscission is linked to carbohydrate and GA concentrations.

Fruit load in citrus is known to be controlled by metabolite availability in the tree through a "competition mechanism" (Goldschmidt and Monselise 1977). It is currently accepted that in citrus natural fruit abscission during the June drop is highly dependent on carbohydrate (Goldschmidt and Koch 1996). Leaf photosynthesis and photosynthate production are key determinants of carbohydrate status in developing fruits (Buwalda and Smith

1990; Mehouachi et al. 2000; Iglesias et al. 2002). Furthermore, treatments such as defoliation, that reduced carbohydrate availability, result in massive fruitlet abscission (Mehouachi et al. 1995; Gómez-Cadenas et al. 2000), while sucrose supplementation increases fruit retention (Iglesias et al. 2003). Sugars are therefore key factors in the regulation of fruit abscission during the June drop. Recently, we showed that girdling specifically affecting terminal developing fruitlets carrying several leaves reduced abscission rates and increased sugar concentration, suggesting that sugars may also be implicated in the regulation of fruitlet abscission during early fruit growth (Iglesias et al. 2006). The present work shows that girdling at anthesis of branches bearing many flowers certainly delayed early abscission of all fruitlets (terminal and non-terminal fruitlets) and also elevated their sugar concentrations (Figs. 2, 3). Furthermore, it was known that girdling increases starch in leaves (Schaffer et al. 1985; Goldschmidt et al. 1985; Martínez-Cortina and Sanz 1991).

There is also evidence in citrus implicating hormonal control of fruit growth and abscission. Ethylene and ABA, specifically, have been associated with abscission (Gómez-Cadenas et al. 2000; Iglesias et al. 2006), while GAs are thought to be stimulators of fruit development (Talon et al. 1990a, b). Moreover, the present evidence suggests that 13-OH-GAs are essential to limit the early abscission of developing ovaries that naturally occurs shortly after anthesis. Thus, it has been reported that the increase of GA_1 concentration, the bioactive 13-OH-GA in citrus, mainly at anthesis and post-anthesis reduced abscission and stimulated fruit development through the initiation of the cell division period (Ben-Cheikh et al. 1997; Talon et al. 1992). Although the stimulating effect of GA_1 on cell division has not been demonstrated in citrus it is well known that GA_1 operates as a strong activator of cell division in other physiological processes, for instance, the induction of stem elongation (Talon et al. 1991; Talon and Zeevaart 1992). In the work presented here, GA_1 (6 DAA) and the two 13-hydroxylated immediate precursors, GA_{19} and GA_{20} (6–20 DAA), increased in girdled fruitlets, coinciding with the initiation of the cell division period (Fig. 4). Although other 13-OH-GAs including those associated with GA inactivation (Mander et al. 1996), are known to be present in citrus (Talon et al. 1992) they were not analysed, the increase of GA_1 suggest that girdling certainly activated GA action. Also, the transitory increase of endogenous GA_1 concentration appears to be sufficient to delay abscission, since GA_1 in citrus fruitlets are generally high at anthesis and shortly after anthesis, and low thereafter (Talon et al. 1992; Ben-Cheikh et al. 1997). In this work, GA analyses were centred in the period 0–20 DAA that coincides with relative higher concentrations of GAs in citrus fruits and also with the interval when major fruit abscission takes place. The data also suggest that the increased metabolic activities through photoassimilate accumulation induced by girdling lead to a continuous accumulation of GA_1 and GA_{20} at the expenses of GA_{19}. Later on, when metabolic activity decreased, reductions in GA_{20} oxidase and 3-β-hydroxilase activities lead to a marked decrease in GA_1 concentration, parallel to an accumulation of precursors GA_{19} and GA_{20}.

Additionally, the main effect of girdling upon abscission was found early after anthesis, between 12 and 32 DAA. However, as observed in our experiments (data not shown) healing occurs, approximately, 40 days after girdling. At this time, abscission rate increased and thereafter decreased continuously to reach a minimum 69 DAA. Afterwards, moreover, abscission rate is almost nil in citrus fruits (Iglesias et al. 2008). This sequence of events is related to the natural restoration of phloem connexions produced by branch girdling after approximately 1 month (data not shown). The restoration of phloem fluxes should recover the original distribution of photo-assimilates and metabolites among all developing reproductive and vegetative structures (Goldschmidt et al. 1985; Wallerstein et al. 1978). This likely increased "competition" for carbohydrate and GAs required for fruit growth in girdled trees, which results in further fruit abscission. Such abscission ceased when equilibrium was reached and fruit load were adjusted to the tree metabolites availability.

In conclusion, the results presented above established a link in developing citrus fruitlets among the reduction of early abscission rates and the higher concentrations of carbohydrates and GAs induced by girdling. The results also suggest that carbohydrates may be determinants also for early fruit set and that girdling may increase GA concentration.

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