



# Ringling branches reduces fruitlet abscission by promoting *PIN1* expression in ‘Orri’ mandarin

C. Mesejo, A. Martínez-Fuentes, C. Reig, M. Agustí\*

Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Camino de Vera, s/n, 46022, Valencia, Spain

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## ABSTRACT

Ringling branches is a technique which is widely used to increase the yield of *Citrus* cultivars with low parthenocarpic ability. When performed during the physiological fruitlet abscission stage it prevents fruitlet drop and increases the number of fruits harvested. This effect has been related with an increased carbohydrate supply, which requires an enhanced photosynthesis efficiency of leafy flowering shoots. Since ringling also reduces vegetative growth, both the number of shoots and the leaves per shoot, the mechanism by which the carbohydrate supply is increased should be revised. Our results show that ringling carried out at this stage maintains the ability of the ovary for cell division mediated by the availability of carbohydrates, as indicated by an increased *CcCYCA1.1* expression. But this effect is not linked with an increase in  $GA_1$  biosynthesis (*CcGA3ox1* expression), as this occurs during fruit set; hence, hormones other than gibberellin must be controlling the physiological fruitlet abscission in response to ringling. We found that an increased expression of the auxin efflux carrier *CcPIN1* gene suggests that ringling induces the auxin export out of the fruitlet and transport to the abscission zone (AZ-C), thus inhibiting its activation and allowing carbohydrates supply to the fruitlet which, thus, prevents abscission and continues growth.

## 1. Introduction

Girdling has been practiced in the culture of fruit trees for thousands of years (Goren et al., 2003). Although the term refers to a technique by which a ring of bark is removed from the trunk or branch, a less aggressive procedure like ringling, in which a simple cut is made around the circumference of the branch, is as effective as girdling (Agustí et al., 1998; 2005).

In citrus, ringling ensures bud sprouting when performed above the grafted buds (De Scheper et al., 2010), increases fruit size when done at the onset of the cell enlargement stage (Cohen, 1984), mitigates alternate bearing if performed by midsummer in the OFF year (Agustí et al., 1992), and increases fruit set when carried out during bloom (Monselise et al., 1972).

Girdling is profitably used to increase fruit set in citrus cultivars with low parthenocarpic ability by reducing the physiological fruitlet abscission (Monselise et al., 1972). When performed at anthesis, branch ringling increases the concentration of gibberellins  $GA_{19}$ ,  $GA_{20}$  and  $GA_1$  (Mehouachi et al., 2009) and also that of hexoses and starch in developing ovaries of Satsuma mandarin (Rivas et al., 2006; Mehouachi et al.,

2009), Clementine mandarin (Rivas et al., 2007) and mandarin-like hybrids (Rivas et al., 2006; 2007). The role of gibberellin (GA) as a pivotal effector stimulating cell division in the ovary walls and regulating the transition of ovary to developing fruitlet has been reported for citrus (Talón et al., 1992; Ben-Cheikh et al., 1997; Mesejo et al., 2016), and also that of carbohydrates governing early physiological fruitlet abscission (Mehouachi et al., 1995; Gómez-Cadenas et al., 2000; Rivas et al., 2007; Mesejo et al., 2019). But at this stage, the effect of ringling is a transitory effect, delaying early natural fruitlet drop that, nevertheless, increases over time, and cumulative abscission becomes similar in ringed and non-ringed trees with no significant differences in yield (Rivas et al., 2006; Mehouachi et al., 2009).

However, when ringling is performed later, for example, during the physiological fruitlet abscission stage, it prevents additional abscission and significantly increases the number of fruits harvested in most citrus cultivars regardless of their parthenocarpic ability (Rivas et al., 2006). This effect has been related to an increased supply of carbohydrates to the developing fruitlets of ringed branches (García-Luís et al., 1988; Golschmidt, 1999) due to the increase in quantum yield efficiency of photosystem II ( $\Phi_{PSII}$ ) in leafy flowering shoots (Rivas et al., 2007).

\* Corresponding author.

E-mail address: [magusti@prv.upv.es](mailto:magusti@prv.upv.es) (M. Agustí).

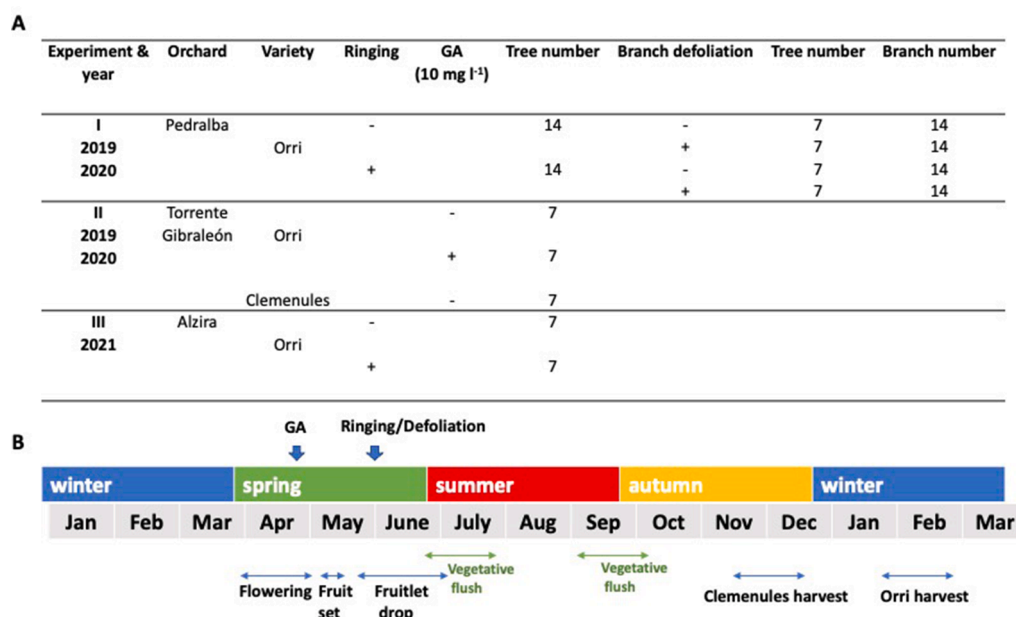


Fig. 1. Experimental design (A) and flow chart (B) containing the phenological events and date of treatments. GA: gibberellic acid.

Table 1

Genes of the *Citrus clementina* v1.0 genome (Phytozome V13, spring 2021, genome ID: 182 • NCBI taxonomy ID: 85681) and primer sequences used in this study.

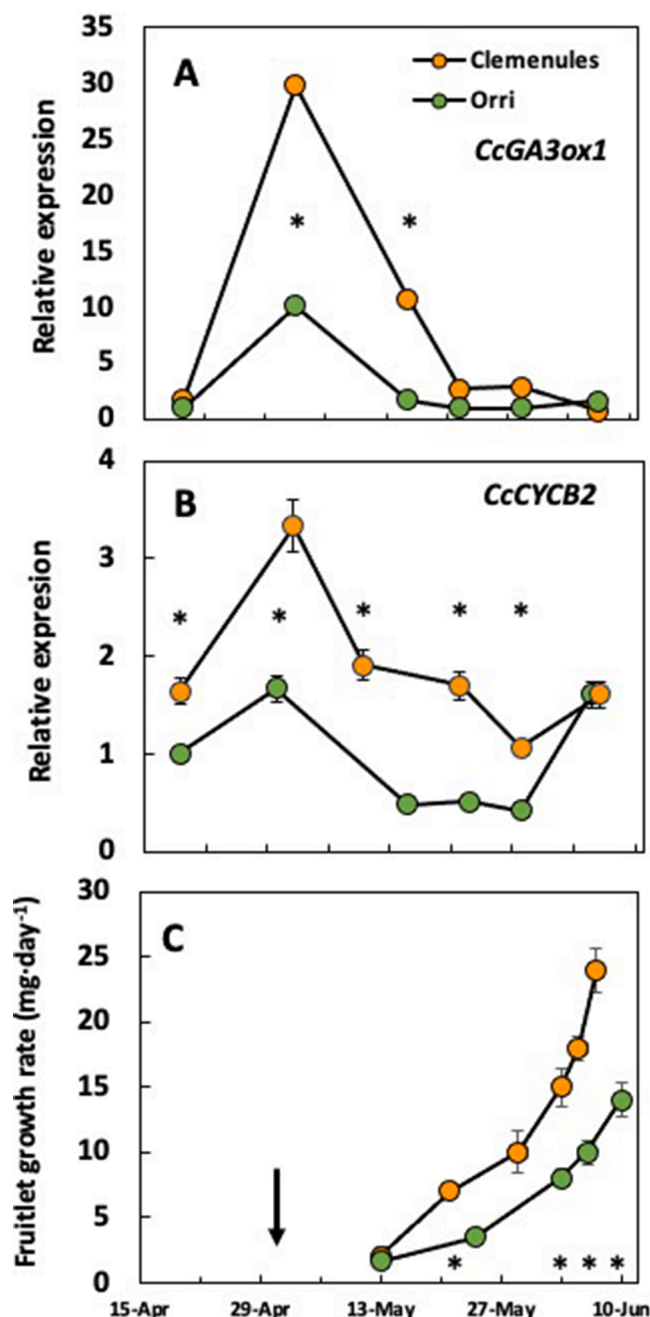
Gen	Description	Id. Phytozome	NCBI Accession number	Forward primer (5'-3') Reverse primer (5'-3')
<i>CYCB2</i>	Cyclin B type. Cell cycle regulator	Ciclev10025564m	XM_024180590.1	GAAAGCAGCAACAGGGAAGC TCCAAAAGAAATTGCGCCGG
<i>CYCA1-1</i>	Cyclin A type. Cell cycle regulator	Ciclev10003984m	XM_006434194.2	CCAGTTTGTGCAACCATTTG TGGACCTCTCCATGAAGTC
<i>CDKB2</i>	Cyclin dependent kinase B type. Cell cycle regulator	Ciclev10002132m	XM_006435405.2	GAGGTCTCCATCTTGCGCAT CGTCTGCCTCCTTGTCT
<i>GA3ox1</i>	Gibberellin 3-beta-dioxygenase. Gibberellin synthesis	Ciclev10027153m	XM_006427062.2	CAACGCAAGATGTCAAATGG CAGCCGGGTAGTAATCAA
<i>PIN1</i>	Peptidyl-prolyl cis-trans isomerase. Auxin transport	Ciclev10026785m	XM_006425453.2	CTCTCTTTGCAGTCCCCTTG CCCTTTGCTCAACTTGCTC
ACTIN	Housekeeper	Ciclev10025866m	XM_006427792.2	TTAACCCCAAGGCCAACAGA TCCTCATAGATTGGTACAGTATGAGAC

Nonetheless, ringing also reduces the number of vegetative shoots and the number of leaves per shoot of vegetative and mixed shoots (García-Luis et al., 1995), hence the hypothesis that its effect enhancing fruit set is only a result of an increased supply of carbohydrates to the ovary should be revised.

When more than one abscission zone (AZ) is present at the peduncle, usually only one is sensitive at a given time (Goren, 1993). This is the case of citrus, in which shortly after anthesis, abscission occurs between the peduncle and the shoot supporting the fruit (AZ-A); GA temporarily controls this early abscission (Ben-Cheikh et al., 1997) and also ringing branches (Monselise et al., 1972). Later on, fruitlets shed at the calyx (AZ-C), where different tissues converge, and girdling branches prevents this until the fruit enlargement stage (Rivas et al., 2006). The sequence of events in citrus fruitlet abscission was thoroughly reviewed by Goren (1993). The mechanism of source-sink regulation of the physiological link between the carbohydrate status and the intensity of abscission (Gómez-Cadenas et al., 2000; Mesejo et al., 2012), the mapping of potential abscission-related genes (Agustí et al., 2008), and spatiotemporal anatomical and histochemical changes (Merelo et al., 2017) have been also studied in depth.

In apple, ethylene promotes the abscission process, whereas auxin (AX), produced in fruitlets and transported basipetally in a polar manner through the AZ, slows it down (Bangerth, 1989; 1997). In *Arabidopsis*, AX transport and distribution are regulated by several proteins, such as *PIN-FORMED 1* (*PIN1*) which acts as a transmembrane component of the AX efflux carrier (Gälweiler et al., 1998). The localization of this protein predicts the AX transport route, and its expression pattern in the fruit follows the AX flow in the pedicel (Blilou et al., 2005). Further, its expression level is higher in mature citrus fruits that do not tend to abscise compared to those that abscise (Mesejo et al., 2021). Thus, the possibility that AX export from the fruitlet through the AZ-C regulates the effect of ringing by reducing its physiological abscission in citrus deserves to be studied in more detail.

To illustrate the GA content-cell division and abscission relationships, the expression of the *CcGA3ox1* gene, which regulates bioactive GA<sub>1</sub> synthesis, and the A- and B-type cyclin, *CcCYCA1,1*, *CcCDKB2* and *CcCYCB2*, which control the transition from the G2 phase into mitosis of the cell cycle, were analyzed. Expression of the *CcPIN1* gene in the ovary, which controls IAA efflux from the ovary to the AZ, was also examined.



**Fig. 2.** Expression pattern of the *CcGA3ox1* (A) and *CcCYCB2* (B) genes on ovaries of 'Orri' and 'Clemenules' mandarin throughout the period of fruit set, and early fruitlet growth (C). For genes, data are the mean of three biological replicates and two technical replicates each. Fruit growth rate is the average of 15 fruitlets per tree. Values for Gibrleón orchard. Data are means  $\pm$  SE. In some cases, SE is lower than symbol size. Arrow indicates the date of anthesis. Asterisks indicate significant difference ( $P < 0.05$ ).

This article aims to describe the effect of ringing branches preventing fruitlet drop when performed during the physiological fruitlet abscission stage. Our results suggest an auxin polar transport from the fruitlet to the abscission zone as the primary effect of ringing branches preventing fruitlet drop. The study is based on the hormonal and molecular point of view, using 'Orri' mandarin because of its low parthenocarpic ability (Schneider et al., 2009).

## 2. Materials and methods

### 2.1. Plant material and treatments

Experiments (Fig. 1A) were conducted over a 3-year period (2019–21) in 4 commercial plantations of 7 to 10-year-old 'Orri' mandarin trees, a mandarin variety selected from  $\gamma$ -irradiated budwood of the 'Orah' mandarin [Temple (*Citrus temple* Hort. ex Tanaka) Dancy (*C. tangerine* Hort. ex Tanaka), grafted onto 'Carrizo' citrange rootstock (*C. sinensis* [L.] Osbeck x *Poncirus trifoliata* Raf.). Orchards were located in eastern Spain, namely, Pedralba, Torrent (2 orchards, Torrent-1 and Torrent-2) and Alzira (Valencia), and southwestern Spain, specifically, Gibrleón (Huelva), with loamy-clay and loamy-sand soil textures, respectively. In Torrent-1 and Gibrleón, an orchard of 'Clemenules' mandarin (*C. clementina* Hort. ex Tanaka) very close to that of 'Orri' mandarin (<1km), grafted onto c. 'Carrizo', was also used for comparison. Trees were planted at 4m x 5m or 5m x 7 m spacing, depending on the orchard, and selected according to similar size, vigor, crop load and flowering intensity. Drip irrigation, fertilization, pest management, and pruning were in accordance with normal commercial practices.

Ringing was performed on the all-main scaffold branches (5–7 cm in diameter) of 7–14 trees. Harp hooked-bladed scissors were used to make a cut 1 mm-wide completely encircling the branch, without reaching the xylem and not removing any bark. Different trees were used each year. Anthesis was defined as when 70% of the flowers were open. Ringing was applied 35 – 40 d after anthesis, i.e., at full fruitlet abscission (Fig. 1B). A set of 7–14 unringed trees served as controls. In 7 trees of both girdled and control trees, two main branches per tree were completely defoliated at the date of girdling. Another set of 7 trees was sprayed at petal fall with gibberellic acid ( $GA_3$ ) (Fig. 1B), at a concentration of 10 mg l<sup>-1</sup>, using a hand-gun at a pressure of 25–30 atm, wetting the trees to the point of run-off. A non-ionic wetting agent (alkyl polyglycol ether) was added at a rate of 0.01%. Seven trees of 'Clemenules' mandarin were also used in the experiment.

In all the experiments, a randomized complete-block design with single tree plots of 7 replications each was performed.

### 2.2. Fruitlet abscission, fruit-set, yield and growth evaluation

Fifteen days prior to anthesis, four branches per tree, with at least 800 nodes each, chosen at random in all canopy orientations, were tagged and the number of remaining flowers/fruitlets per branch recorded and weighed at 10 d intervals until the end of abscission, and the percentage of fruit-set was calculated. Flowers and fruitlets that dropped were collected by spreading nets (9 m<sup>2</sup>) under the trees and counted at weekly intervals until the end of natural fruitlet drop, thus evaluating the abscission rate. Fruitlets of 'Clemenules' mandarin were also weighed periodically, and the fruit growth rate was calculated.

Fresh weight of fruitlets was determined by harvesting 15 fruitlets per branch and tree, stored at 5°C and transported to the lab for weighing.

For RNA extraction, 5 fruitlets per branch from 3 trees were collected and immediately frozen in liquid N<sub>2</sub>, transported to the lab and stored at –80 °C.

The number of new shoots developed from the spring, summer and autumn flush on the selected branches was periodically recorded (Fig. 1B).

Fruit number as well as yield per tree for each treatment in all the orchards were recorded at harvest (Fig. 1B).

### 2.3. Gene expression analysis

Total RNA was isolated from frozen tissue using the RNeasy Plant

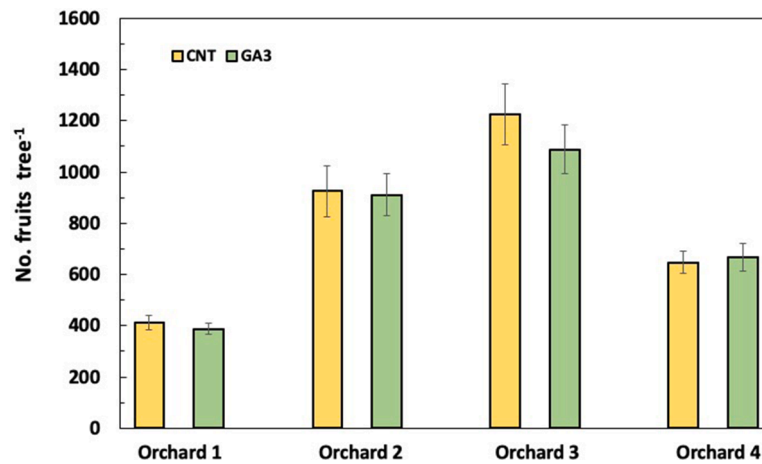


Fig. 3. The effect of gibberellic acid ( $10 \text{ mg l}^{-1}$ ) applied at petal fall on the number of fruits harvested per tree in ‘Orri’ mandarin. Results are the average of 7 trees per treatment, depending on the orchard. Values for Pedralba (1), Torrent-2 (2), Alzira (3) and Gibraleón (4) orchard. CNT: Control. GA3: gibberellic acid.

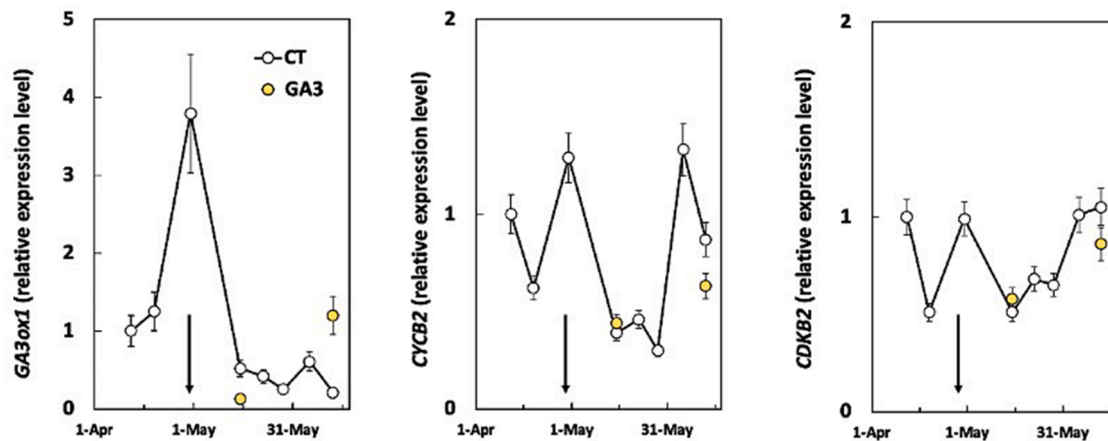


Fig. 4. Relative expression of the *GA3ox1* gene and the B-type cyclin, *CYCB2* and *CDKB2* in fruitlets of ‘Orri’ mandarin treated with  $\text{GA}_3$  ( $10 \text{ mg l}^{-1}$ ) at petal fall. Values for Gibraleón orchard. Data are the means  $\pm$  SE of three biological replicates and two technical replicates each. CT: control. GA3: gibberellic acid.

Mini Kit (Qiagen, Hilden, Germany). RNA samples were treated with RNase free DNase (Qiagen) through column purification following the manufacturer’s instructions. RNA quality was tested by OD260/OD280 ratio and gel electrophoresis. RNA concentration was determined by fluorometric assays with the RiboGreen dye (Molecular Probes, Eugene, OR, USA) according to the manufacturer’s instructions. cDNA was obtained from  $1 \mu\text{g}$  total RNA using the QuantiTect® Reverse Transcription Kit (Qiagen, USA) in a total volume of  $20 \mu\text{l}$ . Quantitative real-time PCR was carried out on a Rotor Gene Q 5-Plex (Qiagen) using the QuantiTect® SYBR® Green PCR Kit (Qiagen). The reaction mix and conditions followed the manufacturer’s instructions with certain modifications. The PCR mix contained  $2.5 \mu\text{l}$  of a 4-fold cDNA dilution,  $12.5 \mu\text{l}$  of QuantiTect® SYBR Green PCR Master Mix (Qiagen),  $1.5 \mu\text{l}$  of  $0.3 \mu\text{M}$  primer F, and  $1.5 \mu\text{l}$  of  $0.3 \mu\text{M}$  primer R, the final volume being  $25 \mu\text{l}$ . The cycling protocol for the amplification consisted of 15 min at  $95^\circ\text{C}$  for preincubation, then 40 cycles of 15 s at  $94^\circ\text{C}$  for denaturation, 30 s at  $60^\circ\text{C}$  for annealing and 30 s at  $72^\circ\text{C}$  for extension. Sequences of the genes studied were obtained from the Phytozome v13 database (<http://www.phytozome.net>, spring 2021). Primers were designed using software Primer 3 web v4.1.0. (<http://primer3.ut.ee/>). The sequences of the primers used are specified in Table 1.

#### 2.4. Statistical analysis

STATGRAPHICS PLUS software was used to analyze the data. Analysis of variance (ANOVA) was performed using Fisher’s least significant difference test for mean separations at  $P < 0.05$ .

### 3. Results

‘Orri’ mandarin has a low parthenocarpic ability and is characterized by a low yield. This is in accordance with its lower *CcGA3ox1* gene expression at anthesis and during the following four weeks when compared to ‘Clemenules’ mandarin (Fig. 2A), a cultivar that also has a low parthenocarpic fruit set. Consequently, since GA reactivates cell division in the ovary wall, the *CcCYCB2* gene expression in the ovary was also markedly low in ‘Orri’ mandarin (Fig. 2B). Hence, ‘Orri’ fruitlet growth rate increased from  $1.5$  to  $10.0 \text{ mg d}^{-1}$  from petal fall (13 DAA) to the onset of cell enlargement (34 DAA), whereas that of ‘Clemenules’ increased from  $2.0$  to  $24.0 \text{ mg d}^{-1}$  during the same period (Fig. 2C).

Therefore, the application of  $\text{GA}_3$  at petal fall might seem logical to increase fruit set of ‘Orri’ mandarin, but the effect was nil (Fig. 3). Furthermore, 15 d after treatment,  $\text{GA}_3$  did not increase *CcGA3ox1*

**Table 2**

Effect of ringing branches on fruit set in *Citrus*. All experiments were conducted on adult trees of commercial plantations, and ringing carried out 30-35 days after anthesis. Values expressed as number of fruits per tree. In all cases differences are statistically significant.

Species and cultivar	Control	Ringed
Sweet orange		
Navelate <sup>1</sup>	436	563
Satsuma mandarin		
Clausellina	250	310
Okitsu <sup>2</sup>	225	315
Clementine mandarin		
Clemenules I <sup>3</sup>	120	230
Clemenules II <sup>4</sup>	387	909
Oronules	94	248
Oroval <sup>5</sup>	481	677
Hybrid		
Nova <sup>4</sup>	892	1096
Fortune I <sup>1</sup>	88	510
Fortune II <sup>1</sup>	339	1086
Ellendale <sup>1</sup>	76	293
Nadorcott I <sup>5</sup>	302	382
Nadorcott II	408	571

<sup>1</sup> Agustí et al., 2003

<sup>2</sup> Rivas et al., 2006

<sup>3</sup> Rivas et al., 2010

<sup>4</sup> Gambetta et al., 2008

<sup>5</sup> Gravina et al., 2016.

expression significantly relative to the untreated control, and 30 d later expression was slightly higher but not statistically significant; similarly, *CcCYCB2* and *CcCDKB2* expression did not differ significantly from the control (Fig. 4).

Another option might be the increase in carbohydrate availability due to ringing branches. This was carried out 35 – 40 DAA, when a general response has been obtained for most citrus cultivars that lack adequate fruit set (Table 2). Ten days after ringing, fruitlet weight of ringed branches (187 mg) was already significantly higher than that of not-ringed ones (142 mg) (Fig. 5A), differences increasing over time up to 625 and 400 mg, respectively, at the end of the studied period (30 d after ringing). This effect inversely correlated with fruitlet abscission rate, which was significantly reduced on all dates recorded up to 20 d after branch ringing (Fig. 5B), and positively with cell division, i.e. *CcCYCA1.1* gene expression in fruitlets ( $r = + 0.9875$ ;  $p < 0.01$ ) (Fig. 6A), but not with GA biosynthesis, i. e. *CcGA3ox1* expression (Fig. 6B). It is logical because at this stage GA biosynthesis is practically non-existent (Fig. 2A), so cell division must be induced by other plant hormones. Interestingly, defoliation reduced *CcCYCA1.1* expression in fruitlets by 40% compared to controls (Fig. 6A) and, consequently, fruitlet growth, 69 and 211 mg 10 d and 30 d after ringing, respectively (Fig. 5A), and fruit set (Fig. 5C), but did not modify the expression of *CcGA3ox1*, which did not differ from the control (Fig. 6B). It should be noted that ringing recovered *CcCYCA1.1* expression of fruitlets (Fig. 6A) and partially fruit set and growth (Figs. 5C and 5A) on defoliated branches.

The effect of ringing increasing fruitlet growth paralleled a reduction in the sprouting rate. Ten days after ringing, the developing shoot rate on ringed trees (1.4 shoots d<sup>-1</sup>) was significantly lower than that of the control ones (1.9 shoots d<sup>-1</sup>) (Fig. 7). This depressing effect also influenced the autumn sprouting, so that the peak of developing shoots recorded late in September averaged 3.4 shoots d<sup>-1</sup> for the control trees and 1.6 shoots d<sup>-1</sup> for the ringed trees (Fig. 7).

It is important to highlight that ringing branches significantly increased *CcPIN1* gene expression in the fruitlet, whereas defoliation significantly reduced it (Fig. 6C). Expression of this gene correlated

negatively ( $r = - 0.9760$ ;  $P < 0.01$ ) with fruitlet abscission, i.e., positively with fruit set. Thus, at the end of physiological fruitlet abscission, control trees set 1.20% of fruitlets, whereas ringed trees set 2.83%, and no fruitlets remained on defoliated trees (Fig. 6C). Ringing also recovered *CcPIN1* expression (Fig. 6C) and fruit set, 1.30%, of defoliated branches (Fig. 5C). Accordingly, the number of fruits harvested per tree was significantly higher in ringed than in control trees (Fig. 5D), and in terms of yield, that of ringed trees resulted in a 3.5-fold increase compared to the control in both orchards, from 7 and 13 kg tree<sup>-1</sup> to 25 and 49 kg tree<sup>-1</sup>, respectively (Fig. 5D).

#### 4. Discussion

Parthenocarpic transition from ovary to developing fruitlet is due to its autonomous ability to synthesize GA<sub>1</sub> during anthesis which stimulates cell division and, thus, the necessary growth rate to set fruit (Talón et al., 1992; Mesejo et al., 2013; 2016). However, in some cultivars, although the expression of *CcGA3ox1*, i.e., the biosynthetic capacity of GA (and even that of some *CcCYC* genes, i.e., cell division rate), is highest at anthesis, it is very low in relative terms, resulting in a low sink capacity of the ovary and, thus, a growth rate insufficient to ensure the fruit set (Mesejo et al., 2016). This is the case of 'Orri' mandarin (see Fig. 1) for which although ovary growth rate increases over time, the lack of set is due to its marked low level of GA and, therefore, cell division rate, during anthesis, compared to 'Clemenules' mandarin, a cultivar that lacks autonomous parthenocarpy (Talón et al., 1992; Rivas et al., 2006; Mesejo et al., 2013).

This low GA biosynthesis may be due to  $\gamma$ -irradiation which, in addition inducing sterility, modifies the 13-hydroxylation pathway elapsing GA biosynthesis with a low expression of *CcGA20ox2* and *CcGA3ox1*, as observed in 'Moncalina' mandarin (Bermejo et al., 2015). Unexpectedly, the application of GA<sub>3</sub> at petal fall did not increase fruit set of 'Orri' mandarin, so an additional effect of  $\gamma$ -irradiation on GA-signaling is hypothesized.

But the process of fruit set also demands large amounts of carbohydrates. So, parthenocarpic cultivars with low ability to synthesize GA and, consequently, to demand carbohydrates, barely set fruit and have low yields, as occurs in 'Orri' mandarin (Holtzman et al., 2005). In these cases, ringing branches is used successfully to overcome the low productivity. Increases between 30% and 45%, depending on the cv., have been achieved (Agustí et al., 2003), the effectiveness largely depending on the date of ringing (Rivas et al., 2006). When performed at anthesis, the temporary delay in fruitlet abscission is linked to a transitory increase in GA<sub>1</sub> concentration and higher hexose and starch concentrations (Mehouachi et al., 2009; Rivas et al., 2006). Therefore, at the cell division stage GA is required for a fruitlet abscission decrease, and carbohydrates to meet energy demand. However, when performed at full fruitlet abscission (35-40 DAA), as in the current study, ringing does not alter GA<sub>1</sub> concentrations, which are very low, but increases carbohydrate availability to the fruitlet and prevents abscission (Monselise et al., 1972), suggesting that early at the cell enlargement stage, carbohydrates are necessary for fruitlets to continue growing. Moreover, ringing counteracts defoliation depressive effects.

But ringing at this stage also reduced the number of summer - developing shoots, as occurs in many fruit tree species (Goren et al., 2003), the fruitlet accumulating higher carbohydrate amounts to the detriment of developing flushes (Martínez-Alcántara et al., 2015), indicating that reproductive and vegetative growth compete for carbohydrates (Goldschmidt and Golomb, 1982). Furthermore, in some species/cultivars parthenocarpic fruit set can be reached only when vegetative sinks are eliminated (Carbonell and García-Martínez, 1980). Accordingly, the hypothesis that the enhanced fruit set by ringing branches is based on the increased supply of carbohydrates to the

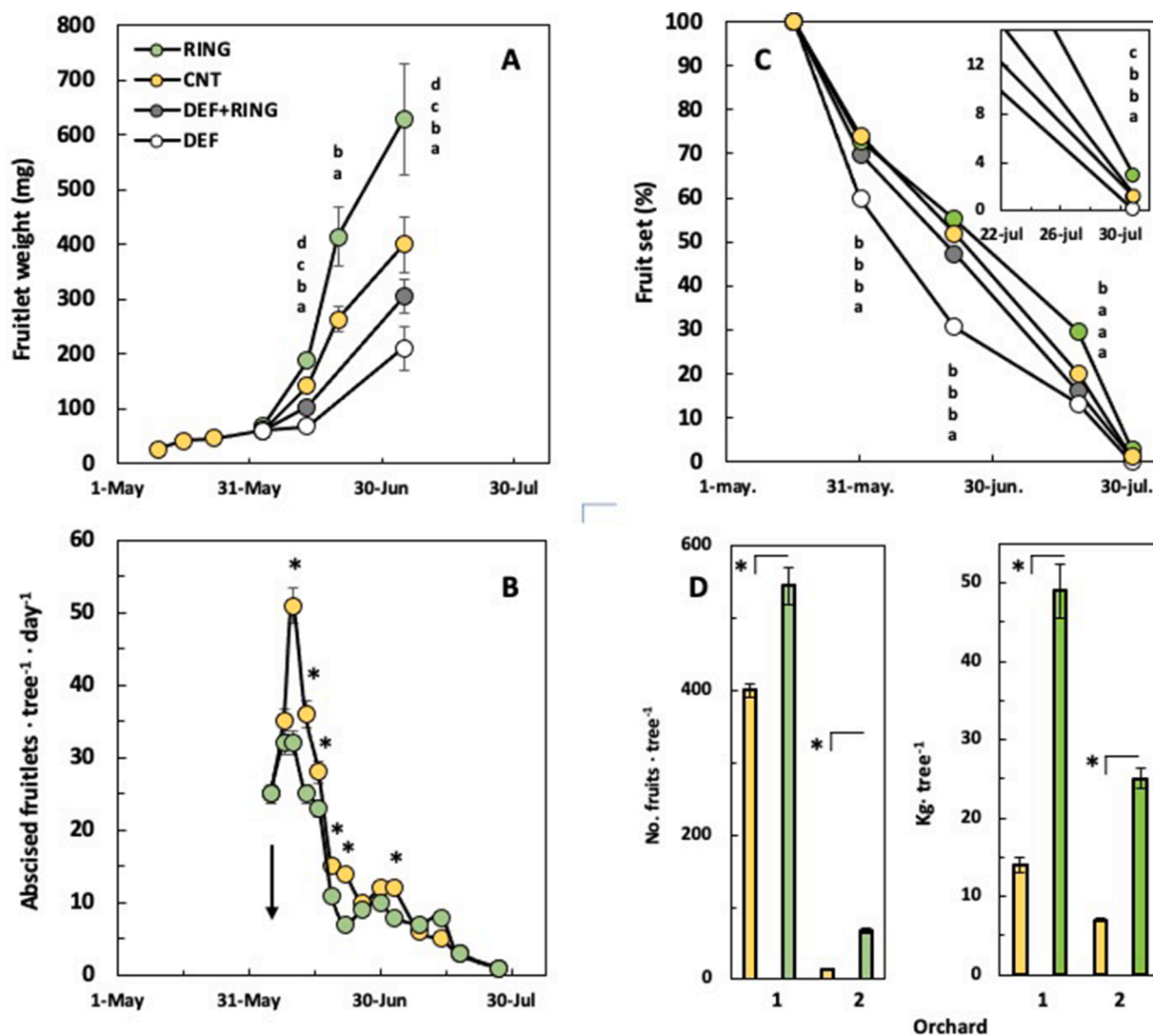


Fig. 5. Effect of ringing branches, defoliation, and their combination on the time-course of fruitlet weight (A), fruitlet abscission (B) and fruit set (C), and that of ringing on the yield per tree (D) of 'Orri' mandarin. Values for Pedralba orchard (A-C) and Pedralba (1) and Torrent-1 (2) orchards (D). Data are means  $\pm$  SE of seven trees. In some cases, SE is lower than symbol size. Different letters and asterisks indicate significant difference ( $P < 0.05$ ). CNT: control. RING: ringing. DEF: defoliation.

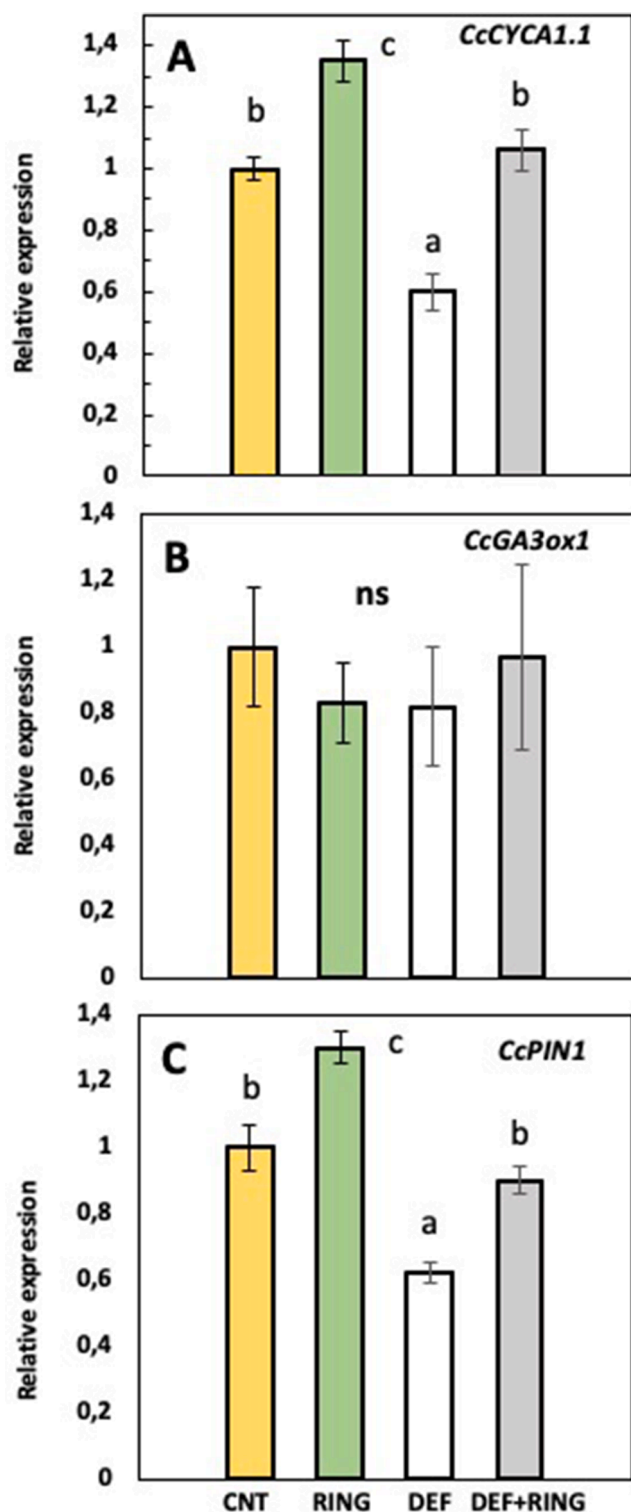


Fig. 6. Effect of ringing branches, defoliation, and their combination on the relative expression of *CcCYCA1.1* (A), *CcGA3ox1* (B), and *CcPIN1* (C) in ovaries of 'Orri' mandarin ten days after ringing. Values for Pedralba orchard. Data are the mean  $\pm$  SE of three biological replicates and two technical replicates each. Different letters indicate significant difference ( $P < 0.05$ ). CNT: control. RING: ringing. DEF: defoliation.

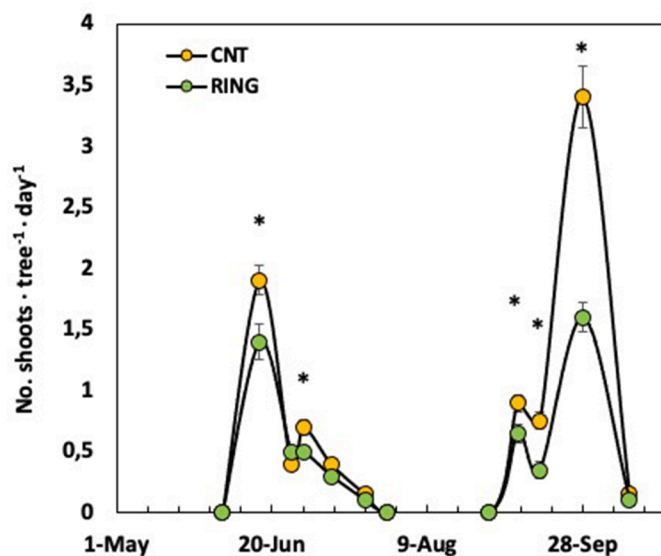
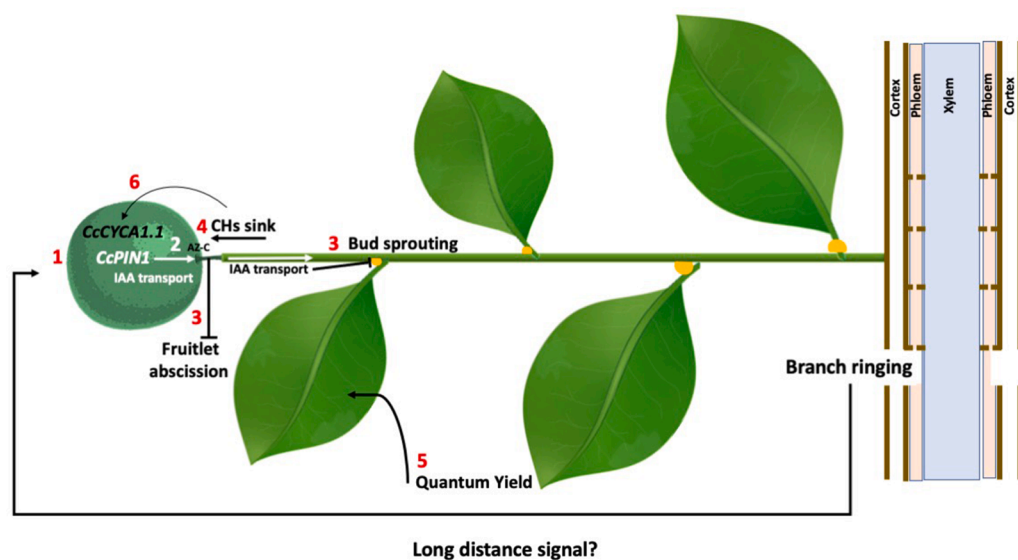


Fig. 7. Effect of ringing branches on summer and autumn sprouting. Values for Torrent-1 orchard. Data are means  $\pm$  SE of seven trees. Asterisks indicate significant difference ( $P < 0.05$ ). CNT: control. RING: ringing.

developing fruitlet (García -Luis et al., 1988; Mataa et al., 1998; Goldschmidt, 1999; Rivas et al., 2006) does not explain, by itself, the effect of ringing branches to prevent fruitlet abscission (Bangerth, 1989). It is accepted that increased fruitlet carbohydrate content by ringing allows it to grow and, thus, it does not abscise, but the reason may be, rather, the opposite: as the ringing prevents abscission, fruitlets can accumulate more carbohydrates and continue growing, thus explaining the greater efficiency of  $\phi_{PSII}$  in the leaves of ringed branches demonstrated by Rivas et al. (2007). Our current study suggests that ringing branches prevents the physiological fruitlet abscission by inducing IAA export out of the fruitlet and transport to the AZ, inhibiting its sensitization and activation, in a manner similar to the "primigenic dominance" proposed for apple (Bangerth, 1989; 2000).

Our results also indicate that ringing branches maintains the ability of the fruitlet for cell division mediated by the availability for carbohydrates. But this does not seem to be linked with an increased GA<sub>1</sub> content for this phenological stage. Firstly, because during physiological fruitlet abscission, no biosynthesis of this hormone occurs (Talón et al., 1990, and our current data), and second, because ringing does not induce their biosynthesis (see Fig. 6B). Therefore, hormones other than GA must be controlling physiological fruitlet abscission.

Takahashi et al. (1975) reported a maximum abscission peak of Satsuma mandarin after the minimum AX content, and Talón et al. (1990) suggested that during the later stages of cell division and early stage of cell enlargement, i. e., the onset of fruitlet abscission, the low parthenocarpic ability of Clemenules mandarin is linked to a high ability to conjugate IAA. This is in accordance to the increased *PIN1* expression (Fig. 6C), that is, the polar transport of auxin (Shimizu-Sato et al., 2009), due to ringing, from the fruitlet to the abscission zone (AZ-C) maintaining the functionality of the vascular bundles (Gälweiler et al., 1998; Aloni, 2010; Merelo et al., 2017; Mesejo et al., 2021) and protecting the fruitlet from abscission (Osborne and Mullins, 1969; Bangerth, 2000; Yuan et al., 2003), so that, i) the fruitlet does not drop, and ii) the carbohydrates can reach the fruitlet, which can continue to grow. The fact that the expression of both *CcCYCA1.1* and *CcPIN1* genes 10 d after ringing correlates positively with ovary growth, and negatively with the percentage of fruitlet abscission, respectively, is consistent with this



**Fig. 8.** Schematic representation of the events that lead to prevent fruitlet abscission by ringing branches during the physiological fruitlet drop. The effect is mediated by polar auxin transport from the fruit to the AZ-C, which maintains fruit sink activity and allows the fruit to continue growing. Numbers indicate the correlative events: 1) ring increases *CcPIN1* expression in the fruitlet, and 2) IAA polar transport across the abscission zone prevents abscission and 3) axillary bud sprouting; 4) fruitlet CHs sink activity increases and, thus, 5) increases photosynthesis to 6) cover fruitlet cell division and growth.

hypothesis, similar to the effect of auxin (2,4-D) treatment delaying mature sweet orange fruit abscission (Mesejo et al., 2021).

In conclusion, ringing branches performed during the physiological fruitlet abscission stops the fruitlet drop and increases the yield in 'Orri' mandarin. Results suggest that the effect is due to the protection of the fruitlet abscission zone (AZ-C) mediated by polar auxin transport from the fruit, which allows the fruitlet to maintain carbohydrate assimilation and continue growing (Fig. 8). In summary, the increase in *CcPIN1* expression and export of auxin plays two complementary roles, reduces abscission and increases fruit sink activity.

#### Authors contribution

C.M. conceived the research and designed the experiments; C.M., A. M.F., C.R. and M.A. performed the experiments; C.M., C.R., A.M.F. and M.A. analyzed and interpreted the data; M.A. wrote the manuscript with contributions from all authors.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data Availability

Data will be made available on request.

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