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Loquat fruit lacks a ripening-associated autocatalytic rise in ethylene production

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Abstract Loquat is considered as non-climacteric fruit; however there is evidence of a climacteric-like maturation. Therefore, it seems its ripening behavior has yet been satisfactory classified. Since autocatalytic regulation of ethylene production during fruit ripening is one of the primary features defining climacteric-like fruit maturation, we examined its ability of autocatalysis during ripening by applying the ethylene-releasing compound ethephon to the on-tree-fruit or ethylene to detached fruit of 'Algerie' loquat and measuring its ethylene and CO_2 production. We also analyzed IAA, gibberellin, cytokinin and ABA content as plant hormones involved in fruit ripening. The fruit response to ethephon (500 mg l^{-1}) applied at color change was immediate producing

increasing amounts of ethylene during the 4 h following the treatment, but 24 h after treatment onwards values were similar to those produced by untreated fruit. Similar results were obtained when applying ethylene to detached fruit (10 µl l^{-1}). Accordingly, applying ethephon (200 mg l^{-1}) did not advance harvest; neither the color nor the percentage of fruit harvested at the first picking date differed significantly from the untreated fruit. Flesh firmness, TSS concentration and acidity of juice were not significantly altered either. IAA concentration reached the maximum value when fruit stopped growing declining sharply at fruit color change; active gibberellins and cytokinins declined continuously during the fruit growth period, and ABA content sharply increased during ripening, peaking after fruit color break. Results indicate that Àlgerie' loquat lacks a ripening-associated autocatalytic rise in ethylene production, and suggest that a decline in gibberellin, cytokinin and IAA concentration might be needed to allow its ripening process to proceed.

Keywords. Loquat · Ripening · Ethylene · CO_2 · Plant hormones

Introduction

Loquat (*Eriobotrya japonica* Lindl.) is a pome fruit whose ripening behavior has yet to be satisfactorily classified. Although considered as non-climacteric fruit (Kader 2002), there is evidence of a climacteric-like maturation. Under Mediterranean environmental conditions, a peak in ethylene production is associated with an increased respiration rate in fruits of five loquat cultivars (Amorós and others 2003). Hirai (1980) also found increased respiration during ripening of the Tanaka cultivar. Analysis of the expression

of ethylene biosynthetic genes revealed an increase in ACC-synthase 2 (*EjACS2*) that paralleled a peak in ethylene production at the time of color change in the Luoyangqing cultivar. The rate of expression of other genes, such as *EjACS1* and *EjACO1*, increased progressively during ripening. These genes, however, did not undergo major changes in detached fruits. These results suggest a climacteric-like maturation of this cultivar regulated mainly by *EjACS2* (Jiang and others 2011).

Other studies revealed that loquat behaves as a non-climacteric fruit since ethylene and CO_2 production gradually declined during fruit maturation in Algerie (González and others 2003) and Mogi (Ding and others 1998) cultivars. In the Golden Nugget cultivar, Undurraga and others (2011) found an increase in ethylene production but not in respiration rate at the time of color break. These authors also reported an increase in the activity of the peroxidase enzyme, but a constant reduction in that of pectinmethylesterase, polygalacturonase and cellulose, and thus they could not confirm that enzyme activity was linked to ethylene production. Accordingly, and although in some cases there is a well-defined peak in ethylene production, there is not enough data available to classify loquat as climacteric fruit. Besides, there is no starch accumulation in the flesh tissues of the Algerie cultivar (Gariglio and others 2002), the firmness of Luoyangqing cultivar is increased during ripening due to tissue lignification (Cai and others 2006), and there is no evidence of a general response to ethephon promoting fruit ripening (Undurraga and Olaeta 2003; Reig and others 2007). Moreover, some reports indicate that after harvest there is a very low level of ethylene production (Blumenfeld 1980; Ding and others 1998; Amorós and others 2003; Cai and others 2006), and others report no concomitant increase in ethylene production and respiration rate (Bumenfeld 1980; Zheng and others 1993; Ding and others 1998; González and others 2003; Kader 2002). These results illustrate the controversy concerning loquat ripening and ethylene

production, and it is risky to conclude that loquat is a fruit with climacteric-like behavior. For further information, see review by Pareek et al. (2014).

Autocatalytic regulation of ethylene production during fruit ripening is one of the major features defining climacteric-like fruit maturation (Yang and Hoffman 1984; Giovannoni 2001). In climacteric fruits, ethylene is able to induce its own biosynthesis through a positive feedback regulation of specific members of the *ACS* gene family and *ACO* genes as well, thus leading to a massive increase in ethylene production triggering the onset of ripening, and all the ethylene-related events occurring during ripening (see reviews by Grierson 2014; Cherian and others 2014). Despite loquat's ability to produce small amounts of ethylene during ripening (Blumenfeld 1980; Ding and others 1998; Amorós and others 2003; Cai and others 2006), the fact that fruit ripening is not stimulated by the ethylene-releasing compound ethephon suggests that autocatalysis of ethylene production does not operate in loquat fruit. However, there is still no evidence of changes in ethylene production and respiration rate in response to ethylene or ethylene-releasing compounds.

Besides ethylene, other plant hormones influence fruit ripening. According to McAtee and others (2013) and Cherian and others (2014), a dynamic interplay between phytohormones and metabolites is required for fruit to mature and ripen. In a number of both climacteric and non-climacteric fruit crops, a reduction in auxin levels is required for ripening to commence (Given and others 1988; Zaharah and others 2012). Furthermore, in fruits for which it is not strictly associated with ethylene, auxin treatment delays ripening (Jones and others 2002), and the initiation of ripening is prevented in transgenic apples, which can maintain high levels of auxin (Schaffer and others 2013). Cytokinins are a powerful antisenescence factor (Richmond and Lang 1957), and there is evidence suggesting that these plant hormones play some role in fruit

maturation (Kumar and others 2014). For example, in loquat, kinetin applied prior to fruit color change significantly delays the loss of chlorophyll (Lou and others 2012). Gibberellins have also been found to delay fruit ripening in both climacteric (Martínez-Romero and others 2000; Singh and others 2007) and non-climacteric fruit (Agustí and others 1981). Finally, an increase in abscisic acid (ABA) has also been associated with color change. In non-climacteric fruit, where no peak of ethylene production during ripening takes place, there is an increase in ABA content (McAtee and others 2013), and any treatment that delays this increase reduces fruit color intensity (Gambetta and others 2014). Therefore, studies integrating the pattern of changes in the most important plant hormones during loquat fruit development and ripening may shed light on its ripening behavior.

The aim of this research is to provide evidence for the behavior of loquat fruit ripening especially that regarding its ability of autocatalysis of ethylene production during ripening. To this end, we used exogenous ethylene and the ethylene-releasing compound ethephon to examine the effect on the fruit inducing ethylene production and respiration rates. We also studied the time-course of the endogenous content of the plant hormones (abscisic acid, indole-3-acetic acid, gibberellin and cytokinin) during the stages prior to fruit ripening (703-709 growth stage on the BBCH-scale; Martínez-Calvo and others 1999) and their role on the initiation of the process (growth stage 801 BBCH-scale).

Materials and Methods

Plant material and treatments

126 Experiments were conducted over four consecutive years (2011-2014) in a commercial
127 orchard located in Callosa d'En Sarriá, Alicante, Spain (38º 39' N, 00º 07' W), and in
128 an experimental orchard at the Universitat Politècnica de València, Spain (39º 28' N;
129 00º 22' W). The 20-25-year-old 'Algerie' loquat (*Eriobotrya japonica* Lindl.) trees
130 were budded onto loquat seedling rootstock, grown in a loamy-clay soil, pH 7.5-8.0,
131 planted 4 x 4 m apart, with drip irrigation (two drippers per tree), and pruned to a vase
132 shape. Fertilization, annual pruning, thinning, as well as pest and disease management
133 were in accordance with commercial practices. The experiments used different trees
134 each year, with no significant differences among years.

135 To evaluate the effect of ethephon (2-chloroethylphosponic acid, Ethrel® 48% sl,
136 Bayer Crop Science, Valencia, Spain) on fruit ripening, 200 and 500 mg l^{-1} were applied
137 at the onset of fruit color change (growth stage 709 on the BBCH-scale), or slightly
138 earlier, to the whole tree with a hand-gun at a pressure of 2.5-3.0 MPa, wetting the tree
139 to the point of run-off. A non-ionic wetting agent (alkyl polyglycol ether) was added at
140 a rate of 0.01%. A randomized complete-block design with single-tree plots of 6-8
141 replications each, depending on the year, was performed. At harvest, the number of
142 fruits harvested per tree was recorded, and commercial fruit characteristics were
143 analyzed. This experiment was carried out in Callosa during the years 2011-2013.

144 In 2014, from early March, when fruit size was about 40% of its final size (growth
145 stage 704 on the BBCH-scale), to senescent fruit [BBCH stage beyond (+) 809] two
146 fruiting shoots per tree bearing at least three fruits each were randomly selected from 6
147 control trees. The fruits, attached to the shoot, were enclosed in hermetic 3-l plastic
148 bottles with a 1.5 cm-diameter rubber stopper (septum). After 2-3 h, a triplicate 1 ml air
149 sample was withdrawn with a hypodermic syringe through the septum for ethylene and
150 CO_2 analysis. Afterwards, the bottle was removed, fruits were aired to allow a free gas

151 exchange with the atmosphere, and fruit color was measured. 3 h later, fruits were
152 detached from the shoot and sealed for another 2-3 h at 20ºC in 1.7 l jar, provided with
153 a septum, for ethylene and CO_2 production analysis. Hence, ethylene and CO_2
154 production was analysed from the same fruit attached to the tree and detached from the
155 tree. This experiment was also performed in Callosa.

156 To evaluate fruit ability of autocatalysis of ethylene production, 200, 500, 750 and
157 1000 mg l^{-1} of ethephon were sprayed on the whole tree during ripening, i.e. at 709, 801
158 and 803 phenological growth stage on the BBCH-scale, using the same surfactant at the
159 same concentration as done in the previous experiment. A set of 15 trees was used for
160 each phenological stage, treating three trees per concentration and using the three
161 remaining trees as controls for comparison. Before treatment and 0.5, 1, 2, 4, 8, 24 and
162 168 h after treatment, 4 fruits per tree were detached from the tree and enclosed in 1.7 l
163 jars for 2 h as above for ethylene production analysis. Besides, ethylene produced by
164 fruits detached 4 h after treatment (when a peak in ethylene production occurred) was
165 also analyzed at 1, 2, 3, 5, 12, and 24 h after detachment.

166 To evaluate fruit response to exogenous ethylene, a set of 60 fruits from 6 untreated
167 trees was sampled at growth stage 801 and 803 BBCH-scale, delivered to the
168 laboratory, and samples of 20 fruits were incubated in an ethylene-free atmosphere (air)
169 or in one of 10 µl l^{-1} ethylene or 1 µl l^{-1} 1-methylcyclopropene (1-MCP), an inhibitor of
170 ethylene action, as described by Lafuente and others (2001). Fruits were incubated in 27
171 l tanks, at 20ºC and 85-90% RH, in the dark, for up to 7 days. To avoid an excess of
172 respiratory CO_2, $Ca(OH)_2$ powder was added to the tanks and fruits were ventilated
173 everyday. On days 0, 1, 3 and 7 of incubation, 5 fruits per treatment were enclosed in
174 jars for ethylene production measurement as described above.

Finally, ten fruits per tree from 3 untreated trees were sampled from early developmental stage (growth stage 703 of the BBCH-scale) to color change (growth stage 801 of the BBCH-scale), frozen immediately with N_2 and stored at –80ºC until gibberellin (GA), indoleacetic acid (IAA), abscisic acid (ABA), and cytokinins (CK) dihydrozeatin (DZ), N^6-(Δ^2-isopentenyl)adenine (iP) and *trans*-zeatin (tZ) content analysis. These last three experiments were performed in 2014 in Valencia orchard.

Harvest and fruit characteristic analyses

Fruit were harvested in accordance with conventional commercial color and size standards. The number of fruits per tree was recorded on each harvest date, and results were recorded as the percentage of fruits harvested on the first picking date. At harvest, 20 fruits per treatment were sampled at random, from all around the canopy at 1.5 m above ground level to evaluate fruit characteristics. Fruit firmness was assessed using a fruit pressure tester FT-011 (Facchini, Italy) with a 1.5 mm-diameter flat cylinder probe. Total soluble solid (TSS) concentration of juice (ºBrix) was assessed with a digital refractometer (Atago, Japan) and free acidity was analyzed by titration with 0.1 N NaOH.

Fruit color was evaluated by determining the *a* and *b* Hunter co-ordinates; three measurements were made per fruit at the equatorial area using a Minolta Chroma Meter CR-300 (Tokyo, Japan).

Ethylene and CO_2 analyses

For ethylene production, a 1 ml gas sample was withdrawn from the headspace of the container and injected into a Trace™ Ultra Gas Chromatograph (ThermoFisher Scientific Inc., Waltham, MA, USA) equipped with a 2m x 1.8 mm alumina column and a flame ionization detector. Nitrogen was used as carrier gas at a flow rate of 30 ml min^{-1} and the column temperature was maintained at 140ºC.

Carbon dioxide concentration inside the containers was determined in a Trace™ Ultra Gas Chromatograph (ThermoFisher Scientific Inc., Waltham, MA, USA) equipped with a Carbowax column and a thermal conductivity detector. Temperature was maintained at 60ºC. The carrier gas was Helium at a flow rate of 45 ml. min^{-1}.

In all cases, gas production values represent the mean of three replicates.

Plant hormones analysis

Frozen material was lyophilized and ground into fine powder. Aliquots (about 50 mg dry matter) of ground material were extracted with 80% methanol containing 1% acetic acid. Internal standards were added and mixed with the aliquots at 4ºC for 1 hour. Deuterium-labelled hormones were used as internal standards for plant hormone quantification.

The extraction protocol was carried out according to Seo and others (2011) with some modifications. In brief, for desaltation, the extracts were passed through reverse phase columns HLB (Waters Cromatografía, S.A., Barcelona, Spain). The plant hormones were eluted by 80% methanol containing 1% acetic acid and consecutively applied to cation exchange MCX columns (Waters Cromatografía, S.A., Barcelona, Spain). The fraction containing the acidic ABA, GAs, IAA hormones was applied through ion exchange WAX columns (Waters Cromatografía, S.A., Barcelona, Spain). The final

residue was dissolved in 5% acetonitrile-1% acetic acid, and the hormones were separated using an autosampler and reverse phase UPHL chromatography (2.6 µm Accucore RP-MS column, 50 mm length x 2.1 mm i.d.; ThermoFisher Scientific Inc., Waltham, MA USA) with a 5 to 50% acetonitrile gradient containing 0.05% acetic acid, at 400 µl min^{-1} during 14 min. To obtain the basic fraction containing cytokinins, the MCX cartridge was eluted with 60% methanol- 5% NH_4OH and the final eluate was dried and dissolved in 5% acetonitrile-1% acetic acid. Cytokinins were separated using an autosampler and reverse phase UPHL chromatography (2.6 µm Accucore RP-MS column, 50 mm length x 2.1 mm i.d.; ThermoFisher Scientific Inc., Waltham, MA USA) with a 5 to 50% acetonitrile gradient during 7 min.

The hormones were analyzed with a Q-Exactive mass spectrometer (Orbitrap detector; ThermoFisher Scientific Inc., Waltham, MA USA) by targeted Selected Ion Monitoring (SIM). The concentrations of hormones in the extracts were determined using embedded calibration curves and the Xcalibur 2.2 SP1 build 48 and TraceFinder programs.

Statistical analysis

Analysis of variance was performed on the data, using the Student-Newman-Keuls' multi-range test for means separation. Percentages were analyzed after arc-sine transformation.

Results

Ethephon applied at 200 mg l^{-1} just prior to fruit color break (growth stage 709 on the BBCH-scale) slightly advanced color development. However, neither the color of the fruit skin at harvest nor the percentage of fruit harvested at the first picking date (early May) differed significantly with regard to untreated control fruit in the three years of the experiment (Table 1). Flesh firmness, TSS concentration and titrable acidity of juice were not significantly affected by the treatment either (Table 1). A higher concentration of ethephon, 500 mg l^{-1} in our experiments, did not influence the response, nor did earlier or later applications (data not shown).

The time-course of ethylene and CO_2 production was not modified by ethephon. In control and 200 mg l^{-1} treated fruit, a well-defined peak of ethylene, similar in amount, was found when fruit changed color (801 BBCH-scale) (Fig. 1A). It is worth noting the very low ethylene production (< 3 nl g^{-1} h^{-1}) found for 'Algerie' loquat. The respiration rate for both control and treated fruit was irregular during ripening, and coincided in time and amount of CO_2 production (Fig. 1B). In both treatments, the maximum CO_2 production was associated with that of ethylene production.

These results reveal a transient effect of both natural or ethephon-induced ethylene production, as shown by the study of hourly ethylene production during 0.5-168 h after treatment by fruit treated with ethephon at color change (growth stage 801 on the BBCH-scale). The fruit response to treatment was immediate, and 30 min after the application of 500 mg l^{-1} ethephon fruit produced significantly higher amounts of ethylene (3.1 nl g^{-1} h^{-1}) than untreated fruit (2.0 nl g^{-1} h^{-1}), the difference increasing over time and reaching a maximum 4 h after treatment (4.7 and 2.3 nl g^{-1} h^{-1}, respectively) (Fig. 2A). But 24 h after treatment, values from treated and control fruit were similar (2.2 and 1.9 nl g^{-1} h^{-1}, respectively) (Fig. 2), and remained almost constant to at least 168 h after treatment (2.1 and 1.8 nl g^{-1} h^{-1}, respectively), when the experiment was

stopped. Furthermore, accumulated ethylene production declined during the 24 h after harvest from 4.7 to 1.2 nl g^{-1} h^{-1} in treated fruit collected 4 h after treatment (r = - 0.989) and from 2.3 to 0.9 nl g^{-1} h^{-1} in untreated fruit (r = - 0.991) (Fig. 3). This kind of response was not dependent on the phenological growth stage at treatment (709 to 803 BBCH-scale) or the concentration applied from 200 to 1000 mg l^{-1} (data not shown). Values from control fruit did not differ statistically over time.

The rate of ethylene production showed a similar trend in both on-tree and detached fruit (Fig. 4A), peaking (1.6 and 1.4 nl g^{-1} h^{-1}, respectively) at color break (12 April; 801 BBCH-scale) (Figs. 4B). However, the respiration rate differed significantly between on-tree and detached fruit (Fig. 4C). On-tree CO_2 production increased until 12 April, coinciding with the peak in ethylene production, remaining almost constant until fruit senescence. In detached fruit, a higher and irregular amount of CO_2 was produced throughout the period studied, ranging 71 – 41 µl g^{-1} h^{-1} (Fig. 4C), with a slight increase also coinciding with the peak in ethylene production (12 April; 801 BBCH-scale), and similar to that found in the previous experiment (see Fig. 1B). Interestingly, a sharp increase in ABA content took place during ripening, peaking after fruit color break, i.e. twelve days after the peak in ethylene production (807 BBCH-scale) (Fig. 4A).

As expected, applying ethylene (10 µl l^{-1}) during ripening to fruit detached from the tree did not increase endogenous ethylene production with regard to air-treated fruit measured 3 d after treatment and up to one week later, regardless the phenological fruit growth stage at treatment (801 or 803 BBCH-scale) (Table 2). A pre-treatment with the ethylene action inhibitor 1-MCP (1 µl l^{-1}) at color break (stage 801 BBCH-scale) did not modify ethylene production either, but when applied at early ripening (stage 803 BBCH-scale) ethylene production of treated fruit 1 day after treatment was significantly

297 higher (4.7-fold) than that of untreated fruit. This difference lessened over time, but
298 lasted for at least 7 days after treatment (Table 2).

299 To elucidate the potential involvement of plant growth regulators other than ethylene
300 in the ripening process of loquat fruit, the endogenous concentrations of IAA and CK as
301 well as GA-biosynthetic and catabolic intermediates during fruit growth and ripening
302 were also determined. Indole-3-acetic acid concentration remained almost constant
303 during fruit development until stage 706 on the BBCH-scale (56 – 64 ng g^{-1} DW)
304 increasing rapidly afterwards up to 156 ng g^{-1} DW when fruit stopped growing (709
305 BBCH-scale), and declining to values similar to those during the active fruit growth
306 period (65 ng g^{-1} DW) at fruit color change (801 BBCH-scale) (Fig. 5A).

307 Cytokinin concentrations dropped during the active fruit growth period. Particularly
308 noteworthy was that of tZ (Fig. 5B), the most abundant CK in loquat fruit, which
309 decreased from 3.80 to 0.42 µg g^{-1} DW (703 to 705 BBCH-scale), remaining almost
310 constant until fruit stopped growing (709 BBCH-scale) (Fig. 5B). At color change (801
311 BBCH-scale), tZ fruit content was almost undetectable. iP and DZ showed a trend
312 similar to tZ, although with a much lower concentration (Fig. 5C and 5D).

313 The values for the earliest precursor of bioactive gibberellins, GA_{12}, declined
314 dramatically during the fruit growth period, from 14 ng g^{-1} DW at fruit growth stage
315 703 of the BBCH-scale to 0.4 ng g^{-1} DW at growth stage 705 BBCH-scale, remaining
316 close to zero until color break (Fig. 6). The subsequent GA-intermediates in the non-
317 hydroxilated pathway, GA_{15} and GA_{24}, showed a pattern similar to that of the
318 downstream bioactive GA_4, while the GA_{15} concentration was considerably lower. The
319 precursor GA_9, however, failed to show the same declining profile, as it decreased
320 slightly from 8.7 ng g^{-1} DW (growth stage 703 BBCH-scale) to 7.5 ng g^{-1} DW (706
321 BBCH-scale), and then dropped sharply until fruit color break (< 0.05 ng g^{-1} DW, 801

BBCH-scale) (Fig. 6). The concentration of the GA_{51}- and GA_{34}-catabolite intermediates was below the detection level. The GA-intermediates in the 13-hydroxilation pathway, GA_{53} and GA_{44}, also declined sharply from fruit growth stage 703 to 705 BBCH-scale, followed by an almost constant value, close to zero, until fruit changed color (Fig. 6). The values of GA_{19} were high during the entire period of fruit growth, ranging 2.8 - 3.6 ng g^{-1} DW from growth stages 703 to 709 on the BBCH-scale (Fig. 6), declining sharply afterwards to 0.8 ng g^{-1} DW at fruit color break (growth stage 801 BBCH-scale), whereas GA_{20} (the precursor of GA_1) declined continuously during the period studied (Fig. 6). The bioactive GA_1 slightly reduced content from growth stage 703 (3.2 ng g^{-1} DW) to 705 BBCH-scale (2.8 ng g^{-1} DW), dropping sharply one week later (1.5 ng g^{-1} DW), and declining somewhat again until fruit stopped growing (1.1 ng g^{-1} DW) and changed color (0.7 ng g^{-1} DW) (Fig. 6). GA-catabolic intermediates showed similar endogenous concentration profiles, although differing in their concentrations, being higher for GA_{29}. Both GA_{29} and GA_8 declined dramatically from 703 to 706 BBCH-scale, the former from 1.8 to 0.8 ng g^{-1} DW, the latter from 4.2 to 0.0 ng g^{-1} DW, and remaining almost constant up to fruit color change (Fig. 6).

To study the role of active GA catabolism, the accumulation rates were calculated for the GA_1 and its precursors and catabolites, based on their concentrations, during the active fruit growth period and at the onset of ripening. Rates of precursors GA_{19} and GA_{20} became negative as fruit completed growth and changed color. The bioactive GA_1 showed the same pattern (Table 3). However, GA-catabolic intermediates revealed an opposite trend, remaining close to zero but positive ($\leq$ 0.005 ng g^{-1} DW day^{-1}) for GA_{29} and being nil for GA_8.

Discussion

In loquat, there is some controversy concerning the climacteric or non-climacteric behavior of fruit at ripening, and despite having been considered as a non-climacteric fruit, there are physiological (Amorós and others 2003) and molecular evidence (Jiang and others 2012) suggesting a climacteric-like ripening behavior of some cultivars. This research contributes to our knowledge on this subject showing that 'Algerie' loquat fruit lacks the ability of autocatalysis of ethylene production, which is an essential feature characterizing climacteric fruit.

Climacteric fruit differs from non-climacteric fruit in its increasing respiration and ethylene biosynthesis rates during ripening (Brady 1987; Lelievre and others 1997). In climacteric fruit, ethylene triggers the onset of ripening, and as a consequence there is a massive increase in ethylene production (Peacock 1972), i.e. its action is continuously required for the progress of the ripening processes. Moreover, exposuring the fruit to ethylene triggers endogenous ethylene biosynthesis and the autocatalytic ethylene production is stimulated (Yang 1981). Some of these aspects have been observed in loquat ripening behavior, but others have not. For example, the peak in ethylene production prior to color break reported for some loquat cultivars (Amorós and others 2003) is not consistent with an increase in respiration rate (Zheng and others 1993; Ding and others 1998; Amorós and others 2003; González and others 2003; Undurraga and others 2011). Our results agree with those reported previously, both for ethylene production and respiration rate, even after applying ethephon.

The release of ethylene produced by ethephon when applied to 'Algerie' loquat just prior to color break did not induce an over-production of endogenous ethylene by the fruit, as expected for a climacteric fruit (McMurchie and others 1972; Downs and others 1991; Yamane and others 2007). Moreover, the patterns of ethylene production and

372 respiration rate in ethephon-treated were similar to those of untreated fruit, suggesting
373 that ethylene released by ethephon might be a transient effect incapable of triggering the
374 autocatalytic biosynthesis of ethylene and, thus, the ripening process. In fact, the
375 application of ethephon at the time of peak of ethylene, i.e. growth stage 801 on the
376 BBCH-scale, increased transiently ethylene production, with a maximum being reached
377 after 4 h and declining gradually to 24 h, revealing that ethephon reached the pulp and
378 releases ethylene, but fruit fails to induce ethylene production, as non-climacteric fruit
379 does. As for ethylene (Lurie and Klein 1989), the upsurge in respiration rates after
380 applying ethephon seems a transient event as well. Accordingly, it may be argued that
381 other ethylene-independent regulatory factors, probably upstream of ethylene, may be
382 responsible for the control of ripening (Leng and others 2014).

383 The over-production of ethylene by fruit treated with 1-MCP at early ripening
384 reinforces the non-climacteric ripening behavior of 'Algerie' loquat. Sisler and others
385 (1996) showed that 1-MCP binds irreversibly to ethylene receptors and, thus, inhibits
386 many ripening-related events such as fruit firmness, color change, aroma, etc., and even
387 blocks the normal autocatalytic rise in ethylene production of climacteric fruits (Sisler
388 and Blankenship, 1993) by down-regulating both *ACC synthase* and *ACC oxidase* genes
389 (Grierson 2014). But for fruits in which ethylene inhibits its own production, such as
390 *Citrus* fruits, 1-MCP eliminates the negative feedback control of ethylene biosynthesis
391 and it is over-induced (Mullins and others 2000; Lado and others 2014). Our results for
392 loquat agree with this, and fruit treated with 1-MCP during ripening over-produced
393 ethylene whereas those treated with ethylene did not, which further reinforces the
394 hypothesis of an auto-inhibition of ethylene production by loquat, the opposite of that
395 expected for climacteric fruit.

Nevertheless, in our experiments, this transient production of ethylene by applying ethephon advanced loquat fruit coloration slightly, but not significantly, suggesting that ethylene may play a role at specific stages of ripening, and this effect is shared with non-climacteric fruits, such as *Citrus* fruits (Purvis and Barmore 1981). This effect, which is common to most climacteric and non-climacteric fruits, has been interpreted by Giovannoni (2001) as an additional regulatory strength of climacteric fruit maturation in addition to general ethylene biosynthesis and signaling. Hence, some ripening regulatory genes may be operating separately from and in addition to ethylene, representing regulatory mechanisms common to both climacteric and non-climacteric fruit species (Giovannoni 2004). In accordance, Undurraga and others (2011) found that cellulose, pectinmethylsterase and polygalacturonase activity did not change during ripening (from color change onwards) in loquat fruit cv. Golden Nugget, suggesting that the transient production of ethylene did not affect enzyme activities. This last finding agrees with our results for which ethephon did not alter flesh firmness in loquat fruit 'Algerie'.

ABA content follows the increase in ethylene production, when its concentration decreases. The involvement of ABA in the induction of ethylene production and other ripening processes has been inferred from some climacteric fruit such as apple, peach or persimmon, for which a sharp increase in ABA accumulation precedes ethylene production and its inhibition delays color change and fruit firmness (Leng and others 2014). By contrast, in non-climacteric fruit ABA deficiency may cause a delay in different ripening related processes (Rodrigo and others 2003). Then, the involvement of ABA in the ripening of loquat is not clear, as it may be the result and not the cause of the process since ABA is the last product in the carotenoid biosynthetic pathway, and may accumulate as carotenoid concentration increase paralleling fruit coloration. It is

421 also likely that in loquat ethylene may induce ABA accumulation, as in the non-
422 climacteric fruit sweet orange (Rodrigo and others 2006), explaining the different
423 timing in the peak of each hormone. Hence, the role of ABA in the ripening process of
424 loquat fruit, as for non-climacteric *Citrus* fruits, is not well understood and there is no
425 evidence as to whether the ABA triggers the process or simply parallels color
426 development (Zhang and others 2009; Gambetta and others 2014).

427 Other plant hormones besides ethylene are involved in the regulation of fruit ripening.
428 For example, in sweet orange, a non-climacteric fruit, fruit changes color by reducing
429 active GA concentrations in the exocarp (Gambetta and others 2012), and a sudden
430 decline in GA-like activity coincides with fruit ripening in Satsuma mandarin (Kuraoka
431 and others 1977; García-Luis and others 1985), indicating that the presence of GA in the
432 exocarp prevents fruit coloration, and may explain why the application of gibberellic
433 acid delays the process in *Citrus* sp. (El-Otmani and others 2000). When applied prior
434 to color break gibberellic acid also delays ripening and increases flesh firmness of
435 persimmon (Ben-Arie and others 1986), mango (Khader 1991), apple (Looney and
436 others 1992), and peach (Southwick and others 1995) among other fruits.

437 In our experiments, the time-course of GA content in developing loquat fruit 'Algerie'
438 shows, an average, a continuous decline until color change for both non-hydroxylation
439 and 13-hydroxylation pathway. This result agrees to that reported for sweet orange
440 (Gambetta and others 2012), and suggests that such a reduction is required for the fruit
441 to change color. The remarkable decline in the bioactive GA_1 during fruit growth and at
442 color break showed a pattern similar to that of GA-intermediate GA_{53}, GA_{44} and GA_{20},
443 indicating that these early biosynthesis steps regulate the GA_1 levels. The different
444 GA_{19}-intermediate accumulation profile suggests that this is subjected to turnover by
445 enzymatic activity in the bioactive GA_1, and the accumulation of GA_{29} together with the

dramatic decrease in GA_8 when levels of GA_1 are declining leads us to examine in more detail the role of GA catabolism in the time-course of GA_1 at the onset of fruit color change. The study of the active GA catabolism and the bioactive GA_1 accumulation rate shows i) accumulation rate of GA_1 became significantly negative at the end of the fruit growth and at fruit ripening, ii) the accumulation rate of the precursor GA_{20} showed a pattern similar to the downstream GA_1, suggesting that this is an active precursor not subjected to turnover in the bioactive GA_1, iii) the sharp decline of GA_8 and the relative constant level of GA_{29} during growth stage 709 to 801 BBCH-scale suggest that catabolism does not play a role in maintaining GA_1 levels, and that during fruit ripening, once the cell stops growing and no dilution by cell growth occurs, active export of GA_1 takes place rather than catabolic activity, in accordance with results reported for fruit color change in other non-climacteric fruits (Gambetta and others 2012).

Active cytokinin, mainly tZ, and in a lower proportion, iP (DH content is extremely low), showed a similar declining profile during fruit growth and ripening. However, iP content declined close to undetectable levels from growth stage 705 BBCH-scale onwards indicating that its activity may be likely constitutive, and probably plays a minor role in ripening. tZ completely declined to be almost nil when fruit stopped growing (709 BBCH-scale) suggesting that it could be involved in the process. Kinetin applied prior to fruit color break significantly delayed coloration in loquat (Lou and others 2012).

The delay in fruit color development by gibberellin and cytokinin is due to the delay of chlorophyll degradation and the carotenoid biosynthesis (Agustí and others 1981; Gross and others 1984; Trebitsch and others 1993) thus maintaining temporarily the tissues in a relative juvenile stage. Actually, GA regulates chloroplast division and

grana stacking through DELLA repressors (Jiang and others 2012). Besides, many physiological and biochemical responses to ethylene can be counteracted by gibberellin and cytokinin (Goldschmidt and others 1977) by reducing the tissue's sensitivity to ethylene (Ben-Arie and others 1989). Therefore, a decline in gibberellin and cytokinin concentration in the fruit to very low values prior to the ripening process seems to be necessary for the fruit to change color. In this way, some kind of balance between ethylene production and the reduction of gibberellin and cytokinin concentration might be responsible for the ripening process in loquat.

The increase in IAA concentration during fruit growth (from stage 703 to 709 BBCH-scale) is in accordance with its role promoting fruit cell elongation (Rayle and Cleland 1972). The decline prior to color break agrees with low auxin concentration required for the initiation of ripening (Manning 1993), which is consistent with the effect of auxin inhibiting the expression of ripening-related genes (Manning 1994). It has been proposed that timing for the onset of ripening might be modulated by changes in auxin biosynthesis and activity (Paul and others 2012).

In conclusion, 'Algerie' loquat fruit has a limited ripening-related response to exogenous ethylene since it lacks a ripening-associated autocatalytic rise in ethylene production, reinforcing the concept that its ripening may be classified as non-climacteric. Nevertheless, small amounts of endogenous ethylene production seem to be required to trigger the ripening process. Besides, the results suggest that a decline in gibberellin, cytokinin and IAA concentration might be needed prior to allow the ripening process to proceed.

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