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RNA isolation from loquat and other recalcitrant woody plants with high quality and yield

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ABSTRACT

RNA isolation is difficult in plants that contain large amounts of polysaccharides and polyphenol compounds. To date, no commercial kit has been developed for the isolation of high-quality RNA from tissues with these characteristics, especially for fruit. The common protocols for RNA isolation are tedious and usually result in poor yields when applied to recalcitrant plant tissues. Here an efficient RNA isolation protocol based on cetyltrimethylammonium bromide (CTAB) and two successive precipitations with 10 M lithium chloride (LiCl) was developed specifically for loquat fruits but it was proved to work efficiently in other tissues of loquat and woody plants. The RNA isolated by this improved protocol was not only of high purity and integrity (A260/280 ratios ranged from 1.90 to 2.04; A260/230 ratios were >2.0), but also of high yield (up to 720 µg on average [CV 21%] total RNA per gram fresh tissue). The protocol was tested on loquat fruit (different stages of development, postharvest, ripening, bruising), leaf, root, flower, stem and bud; quince fruit and root; grapevine cells in liquid culture; and, rose petals. The RNA obtained with this method is amenable to enzymatic treatments and can be efficiently applied for research on gene characterization, expression and function.

Keywords: RNA isolation, fruits, polyphenols, polysaccharides, woody plants.

Introduction

Isolation of high-quality RNA from plants is critical for subsequent molecular experiments such as reverse transcription polymerase chain reaction (RT-PCR), rapid amplification of cDNA ends (RACE), Northern hybridization, and microarray analysis [1-4]. RNA isolation from recalcitrant plant tissues has been problematic due to the presence of the rigid cell walls, large amounts of tannins [5], pigments [6], polysaccharides [7, 8], polyquinones [1, 9], or other secondary metabolites [10, 11]. Moreover, the isolation and purification of RNA from plant tissues, particularly of fruit, has been hindered by the presence of abundant compounds (polysaccharides, polyphenolic compounds, proteins and genomic DNA contamination) that interact with nucleic acids, forming insoluble complexes. These interfering chemicals can cause degradation and low yield of functional mRNA through mechanisms such as oxidation of polyphenols and coprecipitation with polysaccharides [12]. Although common methods have been developed for total RNA extraction from plant tissues, they are often not applicable for a wide range of plant species, especially of woody plants [1, 13]. Some of the protocols described for RNA isolation of plants rich in polysaccharides and polyphenolic compounds have included the use of soluble polyvinylpyrrolidone (PVP) and precipitation with ethanol [14].

The difficulty of isolating RNA from these tissues often requires modifications of existing protocols for developing tissue-specific procedures. Modifications have been reported to the guanidinium-phenol-chloroform method [15] and to the hot borate method [16, 17]; further, pretreatment of lyophilized plant material with acetone has been used to remove polyphenolics from plant tissues [18]. Nevertheless, the yield of RNA obtained

with these methods was inferior to the average yield of the method presented here. Furthermore, various methods based on cetyltrimethylammonium bromide (CTAB) have been developed for tissues containing high levels of polysaccharides and phenols [19-23]; however, these methods are time consuming, technically complex and (in some cases) result in low RNA yield. Recently, Tong *et al.* (2012) reported a method that combines CTAB and phenol/chloroform/isoamyl alcohol (for extraction) and NaCl and LiCl (for precipitation) for improved yields of RNA from different peach tissues [24].

As an initial step of our molecular studies in loquat (*Eriobotrya japonica* Lindl.) we tried to extract high quality RNA from fruits using two commercial reagents (TRIzol®, Life Technologies; and, easy-BLUE™, Intron Biotechnology) and two published protocols for RNA isolation from recalcitrant plant tissues [20, 25]. However, the commercial methods were unsuitable and the results obtained with the published methods were clearly unsatisfactory, even with modifications.

In this study, we present an improved phenol-free CTAB-based procedure for RNA isolation specifically adapted to fruits and tissues with different extent of water content from loquat which is a recalcitrant woody plant rich in polysaccharides, polyphenolics and other interfering substances. The protocol includes a washing step as in Hu *et al.* (2002) [20] and introduces modifications to the grapevine RNA isolation procedure by Reid *et al.* (2006) [25] to increase RNA yield and minimize contamination with polysaccharides and polyphenols compounds. We demonstrate that high quality and quantity of RNA can be obtained systematically from different organs and tissues of loquat in different stages of development or stress conditions. We also show that the method is applicable to tissues of

other woody plants such as *Cydonia oblonga* (quince), *Vitis vinifera* (grapevine) and *Rosa chinensis* (rose). The purity and suitability of the extracted RNA for molecular studies such as gene cloning and expression were demonstrated by RT-PCR of several nucleotide sequences, including a polyphenol oxidase gene fragment from loquat [26], an orcinol O-methyltransferase complete gene from rose [27], an EF1- α -like gene fragment [28] and an actin gene fragment [29] both from grapevine.

Materials and methods

Plant Materials

Loquat (*Eriobotrya japonica* Lindl.) fruit at different stages of development and ripening, as well as leaves, stems, buds, flowers and roots, were harvested from Cooperativa Agrícola Callosa d'en Sarrià (Alicante, Spain) and a private orchard in Seville (Spain) in 2011 between March and May. Postharvest conditions were attained by storage at room temperature (for up to 21 days) of intact fresh fruits that were detached at optimal harvesting time. Fruit bruising was attained by hitting intact fresh fruits detached at optimal harvesting time with a sphere of 50 g in free fall from a height of 20 cm. Each fruit was beaten in four different positions for most of the flesh to be affected by bruising and allowed to stand for up to 144 h at room temperature. Green fruits and roots of *Cydonia oblonga* (quince) were harvested from a private orchard in Benidorm (Alicante, Spain). Grapevine (*Vitis vinifera* cv. Gamay) suspension cells which had been elicited with either cyclodextrins, methyljasmonate or both in combination were prepared in our laboratory as described elsewhere [30, 31]. Young and adult petals of *Rosa chinensis* (rose) were collected

from the Alicante University campus gardens (Alicante, Spain). The tested plant species, tissues and sample treatments used for RNA isolation in this work are summarized in Table 1. All tissues were processed (sliced, minced or portioned), immediately frozen in liquid nitrogen and stored at -80 °C until used.

Sample Pretreatment

High water content tissues, including loquat and quince fruits as well as grapevine cells, were lyophilized for 48 hours. The other tissues and organs tested were processed directly for RNA isolation.

Solutions and Reagents

All reagents used were of molecular biology grade. Diethyl pyrocarbonate (DEPC)-treated water (DTW) was used for all solutions. Plastic and glassware were immersed in 0.1% (v/v) DTW at 37°C overnight, then autoclaved at 121°C to inactivate RNases.

Washing buffer [20]. 100 mM Tris-HCl pH 8.0; 0.35 M sorbitol; 10% (w/v) PEG 6000; 2% (v/v) β -mercaptoethanol (added just before use). The solution excluding Tris-HCl was DEPC-treated and Tris-HCl was added after autoclaving.

Isolation buffer [25]. 300 mM Tris HCl pH 8.0; 25 mM EDTA; 2 M NaCl; 2% (w/v) CTAB; 2% (w/v) PVPP; 0.05% (w/v) spermidine trihydrochloride; 2% (v/v) β -mercaptoethanol (added just before use). Note that, during storage at room temperature,

some precipitation may occur; thus, the reagent has to be stirred sufficiently to dissolve precipitated ingredients before using.

Other reagents. 10 M LiCl; 3 M sodium acetate (NaOAc) pH 5.2; DTW; absolute ethanol (EtOH); 70% (v/v) ethanol; and Chloroform-isoamylalcohol (24:1, v/v) (Chl:Iaa).

RNA isolation protocol

Grinding step. One gram of plant tissue (lyophilized tissue or freshly frozen material) was placed in a pre-cooled mortar. The samples were ground to obtain a fine powder in liquid nitrogen and quickly transferred into a sterile disposable polypropylene tubes while frozen and further kept in liquid nitrogen until all samples were prepared.

Washing step. This step was introduced only for highly hydrated tissues such as loquat fruit or quince fruit flesh and grapevine cell suspension, and it was performed as in Hu *et al.* (2002) [20] after lyophilization and grinding of the tissues. For other tissues with a lower water content (such as leaves, stems, buds, flowers, roots, young and adult petals), this step was omitted. Washing buffer was added in a proportion of 10 ml per gram of tissue powder, vortexed for 1 min and centrifuged at 3,500 g for 15 min at 4°C. The suspension was decanted and the supernatant and floating cell debris were discarded.

Isolation step. This step was essentially as described in Reid *et al.* [25], but slightly modified by changing the centrifugation conditions. Pre-warmed isolation buffer (10 ml, 65 °C) was added to 1 g of tissue powder (previously subjected to the washing step, if needed),

homogenized by vortexing, and incubated at 65°C (10 min), with shaking every 2 min. The mixture was extracted with an equal volume of ChI:Iaa, and centrifuged at 5,000 g (10 min, 4 °C). The aqueous layer was transferred to a new tube, mixed with an equal volume of ChI:Iaa, and centrifuged at 10,000 g (10 min, 4°C), with the aqueous supernatant being transferred to a new tube. NaOAc (0.1 vol. 3 M, pH 5.2) and isopropanol (0.6 vol.) were added and mixed, with the mixture being stored at -80°C for 30 min (alternatively, the supernatant can be stored at -20°C for 1 hour). Precipitated material, including nucleic acids and remaining carbohydrates, was recovered by centrifugation at 20,000 g (20 min at 4°C).

Purification step. Resulting pellets were dissolved in 1 mL DTW and transferred to a microcentrifuge tube. The RNA was selectively precipitated by addition of 0.3 vol of 10 M LiCl, mixing and incubation on ice for 90 min, then followed by centrifugation at 20,000 g for 30 min at 4°C. This step was repeated twice. Alternatively, if RNA is precipitated overnight at 4°C, a single 10 M LiCl precipitation may be sufficient to obtain high quality RNA. The RNA pellet was resuspended in 0.1 ml DTW, and 0.1 vol of NaOAc (3 M, pH 5.2) and 2 vol of cold absolute ethanol were subsequently added and immediately centrifuged at 20,000 g for 20 min at 4°C. The new pellet was washed with iced-cold 70% (v/v) ethanol, let to dry, and dissolved in a volume of 30-50 µL DTW.

RNA analysis. RNA purity and concentration were assessed spectrophotometrically by determining the absorbance of the samples at 230, 260 and 280 nm and by A260:A280 and A260:A230 ratios. RNA integrity was evaluated by electrophoresis on 1.2% (w/v) agarose gel containing 6% (v/v) formaldehyde after staining with ethidium bromide (EtBr) and

visualization under UV light. The integrity of the RNA samples was also analyzed on an Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip (Agilent Technologies) according to the manufacturer's handbook.

RT-PCR. First-strand cDNA was synthesized from 1 µg of total RNA using a cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific) according to the manufacturer's instructions. To assess the quality of RNA, RT-PCR was performed with forward and reverse primers from the coding region of a loquat polyphenol oxidase gene fragment (Acc AB011830) (*PPO-DB-1F*: 5'-ACGACCAAGCCGGGTTCACAG-3' and *PPO-DB-R*: 5'-TAAGTCAGTTCTCTTACCTCCAAG-3'); from orcinol O-methyltransferase complete gene of rose (Acc AJ439741) (*OOMT-F*: 5'-ATGGAAAGGCTAAACAGCTTTAGACACCTTAAC-3' and *OOMT-R*: 5'-TCAAGGATAAACCTCAATGAGAGACCTTAAACC-3'); from EF1-alpha-like gene fragment from grapevine cv. Merlot (Acc GU585871) (*EF1A-F*: 5'-GAACTGGGTGCTTGATAGGC-3' and *EF1A-R*: 5'-AACCAAAATATCCGGAGTAAAAGA-3'); and from actin gen of grapevine (Acc JQ989220) (*ACT-F*: 5'-CTTGCATCCCTCAGCACCTT-3' and *ACT-R*: 5'-TCCTGGGACAATGGATGGA-3').

Results

Extraction of loquat fruit RNA using established protocols

Prior to the development of an improved method, we tested four protocols for plant RNA isolation: two commercial methods (Trizol®, Life Technologies™, and Easy-Blue™, Intron Biotechnology); and, two published methods for recalcitrant plant tissues (Hu *et al.*, (2002) [20], and Reid *et al.*, (2006) [25]). The commercial methods were carried out as indicated in the manufacturer's handbook; however, no RNA bands were observed in agarose gels (see supplementary information). The methods described in the literature led to diffuse bands and low yield of RNA when applied as described. Application of specific modifications improved results significantly in both cases. Modifications to the protocol of Hu and coworkers included lyophilization of tissue and fragmentation in mortar with LN2, and purification of the precipitated RNA with Nucleospin® column (Invitrogen) to remove carbohydrates. These modifications improved the RNA quality but the yield was low. Modifications applied to Reid and coworkers' protocol included the sample pretreatment as above; washing as in Hu *et al* [20]; and those that lead to the optimized protocol described in Materials and Methods (see supplementary information).

Extraction of loquat fruit and other plant tissues RNA using a robust CTAB-based protocol

High-quality RNA was obtained through the protocol described in this paper. The RNA integrity was assessed by the sharpness of ribosomal RNA bands visualized on a denaturing 1.2% agarose gel. For all RNA samples tested, well-resolved 28S and 18S rRNA bands were observed, with no visible signs of degradation (Figures 2, 3), and also sharp 5S RNA peaks were detected with the Bioanalyzer® (Figure 4).

The yields of total RNA ($\mu\text{g/g}$ fresh weight [FW]) were as follows for loquat fruit: different development stages, ~ 370 - 850 ; and, fruits subjected to ripening, ~ 610 - 760 ; postharvest fruit, ~ 740 - 800 ; and, fruits stressed by mechanic damage, ~ 460 - 730 . In other organs and tissues of loquat, the yield ranged from ~ 630 (young stem) to ~ 850 (young leaf). For quince tissue, yields ranged from ~ 750 (young roots) to ~ 790 (green fruit). For cell suspensions of grapevine, yields ranged from ~ 880 - 950 . Finally, the yield of total RNA for different stages of rose petals was ~ 800 (young) and ~ 825 (adult). For the whole set of samples extracted, the average yield was $720 \mu\text{g RNA/g FW}$ with a coefficient of variation (CV) of 21% (Table 1).

The A_{260}/A_{230} ratio was higher than 2.0 for all the samples (average 2.3, CV 6.1%). This is indicative of a high RNA purity and the absence of contamination with polyphenolic and polysaccharide compounds. The A_{260}/A_{280} ratios ranged from 1.90-1.99 (average 1.94, CV 1%), indicating low or no protein contamination (Table 1), and the RIN values were always above 9 (Figure 4). Overall, these data demonstrate that the extraction protocol described herein was efficient in yielding high quality, integrity and quantity of total RNA from all tissues and conditions tested.

Checking of genomic DNA contamination

DNA contamination may appear as high MW bands in agarose gels but, as observed in Figures 2 and 3, high MW bands of DNA contamination are not visible in our gels. However, when the abundance of contaminant DNA is low, such bands might not be detected. Genomic DNA of contamination in loquat, rose and grapevine RNA samples was

further assessed in control RT-PCR reactions where retrotranscriptase was omitted. As shown in Figure 5, amplification of a 596 bp fragment corresponding to a partial polyphenol oxidase gene from loquat (Acc AB011830), as well as a complete 1104 bp orcinol O-methyltransferase gene from rose (Acc AJ439741), a 164 bp of EF1-alpha-like gene fragment from grape cv. Merlot (Acc GU585871) and 81 bp of actin gene fragment from grapevine (Acc JQ989220), did not occur unless retrotranscriptase was included in the RT-PCR reactions. This was true even though amplifications were done with a large number of cycles, thus indicating absence of any significant genomic DNA contamination. In particular samples, the genes were also not amplified even in fully supplemented RT-PCR reactions, indicating that these tissues do not express the tested gene (as later confirmed by northern blot, unpublished results).

Total RNA from different tissues and organs extracted by this method is not only suitable for most common applications such as RT-PCR shown here, but also for cDNA library construction and northern blot analysis (data not shown). This method was shown to be very robust and thus could be particularly useful for loquat and other recalcitrant plant samples containing high levels of polysaccharides and polyphenolic compounds, as well as for plant tissues with high water content (see simplified scheme, Figure 1).

Discussion

Although presently there are many methods for plant RNA isolation, it is necessary to develop new optimized protocols for different plant species or organs. This is true even for identical tissues at different developmental stages or stress conditions because the

considerable variability in yield and quality of a given method is largely attributable to the composition and content levels of phytochemicals [32, 33]. Polysaccharides and polyphenols are the most difficult contaminant substances to remove in RNA isolation processes as the oxidation of polyphenols and the similarity of physicochemical properties between polysaccharides and RNA lead to the co-precipitation at the RNA precipitation step [12]. Loquat is a recalcitrant woody fruit tree, compared to herbaceous plants, its leaf and floral buds during differentiation contain much more abundant and intricate polysaccharides, proteins and secondary metabolites such as polyphenols [34]. These compounds cause great difficulty for isolating total RNA with high quality and high yield. The high water content of the fruit tissue is an added problem.

Traditional CTAB protocols [19, 35] involve an initial disruption of tissues in standard CTAB lysis buffer, the separation (twice) by phenol-chloroform of the RNA aqueous phase from other cellular compounds (proteins, genomic DNA and polysaccharide residues), and the subsequent precipitation of RNA with lithium chloride and anhydrous alcohol. However, in plants rich in polysaccharides, proteins and secondary metabolites, these conventional protocols produce an inferior quality, low-yield RNA which leads to poor RT-PCR amplification. This is mainly because the repeated extraction with phenol-chloroform is inefficient at removing polysaccharides and proteins [36]; however, there are also large losses of total RNA from having to discard ~20-25% of the supernatant each time.

Besides, polyvinyl pyrrolidone (PVP) can combine irreversibly with the long polyA tail of messenger RNA [36] and, when used jointly with phenol, results in co-precipitation losses [20, 37]. In a modified CTAB buffer, it was shown that including β -mercaptoethanol

as a strong reducing agent prevented polyphenols from oxidizing and also made RNases denature irreversibly [38]. Polyvinyl-polypyrrolidone (PVPP), as an insoluble cross-linked polymer instead of PVP, was shown to combine exclusively with polyphenols to form complexes by hydrogen bonding [20, 37], thus avoiding RNA losses.

The protocol developed here combines the use of optimal agents in a CTAB-based extraction buffer and some critical improved steps. These include the tissue lyophilization and a washing step [20] for highly hydrated tissues as well as two steps of separation of total RNA from polysaccharide and DNA residues with 10 M lithium chloride (LiCl), which is a strong dehydrating agent that promotes specific RNA precipitation [36, 39].

Except for lyophilization, the whole protocol (including two precipitations with 10 M LiCl) can be completed in a working day. Using common equipment of a molecular biology laboratory, eight samples can be routinely handled in parallel. Unlike common protocols [40-46], we eliminate the use of phenol and all centrifugation steps are done in high speed (not ultra-) centrifuge.

As a result, the quality (purity and integrity) and yield of isolated total RNA achieved by our method is very high and reproducible irrespective of the species, tissue and physiological state. As compared to a recent improved protocol based on CTAB, phenol and LiCl for preparation of high quality RNA from peach tissues [23], we obtain similar results in quality (A_{260}/A_{280} ; A_{260}/A_{230}), but significant improvements in yield. In comparable samples of young tissues (e.g., flower, petal, leaf, stem and fruit), the increase is modest (~1.5-fold); however, the increase is substantial (~10-fold) in adult and stressed

tissues of petal, leaf and mature fruit, and even greater (~15-fold) in postharvest storage fruit. As compared to yields obtained by Reid et al. [24] in analogous grapevine tissues (in $\mu\text{g/gFW}$, up to 600 in leaf, up to 300 in flower and root; up to 120 in pre-veraison pericarp; up to 30 in post-veraison pericarp) our improved protocol leads to significantly better results, particularly in fruit pericarp (Table 1). Like grape berries or peach, loquat fruit pericarp also reaches pH values ranging from 2,5 to 4 during development [47], thus our method is expected to give improved results in RNA yield in other fleshy acidic fruits. Therefore, a major strength of our improved method is its robustness irrespective of the type, age and physiological state of the tissue. In addition, it avoids the use of phenol leading to cost-savings and less chemical toxicity. While commercial reagents for RNA extraction may provide high sample throughput, the quality and yield obtained are very sample-dependent, as we have shown here. Our improved protocol proved to be completely suitable for extracting RNA from different tissues and organs of plants rich in polysaccharides, proteins and secondary metabolites, and the RNA was competent for molecular downstream applications such as RT-PCR and Northern blot analysis.

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Figure captions

Fig.1. Shows a simplified scheme of the improved method workflow. For details see Materials and Methods section.

Fig.2. Total RNA from different organs and tissues of from loquat, separated on an agarose gel (1,2%) containing formaldehyde: (lane 1) 100% green fruit; (lane 2) 50% green fruit; (lane 3) 50% veraison fruit; (lane 4) 100% veraison fruit; (lane 5) harvest; (lanes 6-7-8) 7, 15 and 21 days of postharvest, respectively; (lanes 9-10-11) 7, 15 and 21 days of over-ripening on tree, respectively; (lanes 12-13-14) stress conditions of bruised fruits induced by mechanical damage after 24, 48 and 144 hours, respectively. From lane 15 to 19 different organs and tissues of loquat tree: (lane 15) young leaf; (lane 16) terminal bud; (lane 17) flowers; (lane 18) young stem; and (lane 19) lignified root. The percentage (50

and 100%) of the green fruit refers to the size at collected time. At veraison the % refers to the extent of fruit color change (green to yellow).

Fig.3. Total RNA from different tissues of from quince, grapevine and rose, separated on an agarose gel (1,2%) containing formaldehyde: (lane 20) green fruit quince, (lane 21) mature fruit of quince, (lane 22) quince young roots, (lane 23) elicited grapevine cells with cyclodextrins and methyljasmonate, (lane 24) elicited grapevine cells with cyclodextrins and ethanol, (lane 25) grapevine cells without eliciting, (lane 26) young rose petals and (lane 27) adults rose petals.

Fig.4. Total RNA samples analyzed in the Agilent 2100 Bioanalyzer® with the 6000 Nano LabChip. M, Ladder/marker; Lanes 1-2, young and adult petals from rose, respectively; Lanes 3-5, grapevine cells elicited with cyclodextrins and methyljasmonate, elicited with cyclodextrins and non-elicited, respectively; Lanes 6-7, loquat fruit 100% green and ripening at 15 days, respectively; Lanes 8-9, loquat terminal bud and young flower, respectively. The quality of RNA is indicated by the RIN values at each lane.

Fig.5. RNA quality assessment by enzymatic treatment and detection of DNA contamination. Agarose gel separation of PCR products. 1 µg of RNA from different samples was subjected to retrotranscription in the presence (lanes 1, 3, 5, 7, 9, 11 and 13) or absence (lanes 2, 4, 6, 8, 10, 12 and 14) of retrotranscriptase enzyme. 2 µl of the resulting reaction was used as template for PCR with the primer pairs PPO-DB-F/PPO-DB-R (A,B), OOMT-F/OOMT-R, EF1A-F/EF1A-R and ACT-F/ACT-R (C). The resulting products were analyzed after 30 (A), 40 (B) and 35 (C) cycles of PCR. The starting RNA samples

were from 100% green fruit of loquat (lanes 1, 2); loquat fruit in optimal stage of harvest (lanes 3, 4); postharvest loquat fruit (lanes 5, 6); ripening loquat fruit (lanes 7, 8); young petals of rose (lanes 9, 10); cells grapevine elicited with cyclodextrins and methyljasmonate (lanes 11, 12); and grapevine cells elicited with cyclodextrins (lanes 13, 14). The arrow point indicates the expected size for the PCR products. MW: DNA molecular weight marker; GeneRuler 1 kb DNA Ladder, Thermo Scientific (A), DNA Molecular Weight Marker VI, 0.15 – 2.1 kbp, Roche (B) and GeneRuler 100 bp Plus DNA Ladder, Thermo Scientific (C).

Table 1. RNA yield and quality from different organs and tissues of plants with high levels of polysaccharide and polyphenolic compounds using spectrophotometric determinations.

Plants	Sample conditions	Organs and tissue analyzed	Absorbancy Ratios ^a		Yield (µg/g FW) ^b
			OD 260/280	OD 260/230	
<i>Eriobotrya japonica</i> Lindl. (loquat)	Different stages of development of loquat fruit	100% Green fruit*	1.94 ± 0.031	2.54 ± 0.081	374 ± 14.84
		50% Green fruit	1.93 ± 0.025	2.16 ± 0.067	522 ± 11.67
		50% Veraison fruit**	1.90 ± 0.049	2.19 ± 0.042	376 ± 21.54
		100% Veraison fruit	1.94 ± 0.051	2.28 ± 0.081	803 ± 14.73
		Harvest***	1.91 ± 0.026	2.18 ± 0.045	854 ± 34.39
	Postharvest	Postharvest at 7 days [†]	1.98 ± 0.006	2.21 ± 0.044	798 ± 19.67
		Postharvest at 15 days	1.91 ± 0.015	2.50 ± 0.092	794 ± 23.45
		Postharvest at 21 days	1.96 ± 0.053	2.68 ± 0.090	744 ± 40.44
	Stress conditions by ripening	Ripening at 7 days ^{††}	1.93 ± 0.032	2.16 ± 0.055	758 ± 25.65
		Ripening at 15 days	1.94 ± 0.006	2.23 ± 0.064	724 ± 9.29
		Ripening at 21 days	1.95 ± 0.015	2.20 ± 0.050	610 ± 10.69
	Stress conditions of bruised fruits induced by mechanical damage	Bruised at 24 hours ^{†††}	1.99 ± 0.026	2.43 ± 0.076	730 ± 23.45
		Bruised at 48 hours	1.93 ± 0.042	2.31 ± 0.035	623 ± 32.14
		Bruised at 144 hours	1.96 ± 0.015	2.16 ± 0.035	462 ± 27.05
	Leaf	Young leaf	1.93 ± 0.023	2.21 ± 0.046	850 ± 19.07
		Adult leaf	1.94 ± 0.015	2.17 ± 0.030	845 ± 19.30
	Bud	Terminal bud	1.93 ± 0.015	2.17 ± 0.075	755 ± 21.79
	Flower	Young flower	1.94 ± 0.012	2.22 ± 0.036	680 ± 21.73
		Adults flower	1.94 ± 0.012	2.22 ± 0.036	676 ± 18.71
	Stem	Young stem	1.91 ± 0.015	2.18 ± 0.060	633 ± 8.50
		Adult stem	1.92 ± 0.010	2.17 ± 0.070	624 ± 7.09
	Root	Lignified root	1.93 ± 0.012	2.21 ± 0.064	642 ± 7.00
<i>Cydonia oblonga</i> (quince)	Fruit	Green fruit	1.94 ± 0.012	2.46 ± 0.047	790 ± 17.61
		Mature fruit	1.94 ± 0.017	2.20 ± 0.040	782 ± 27.53
	Root	Young root	1.95 ± 0.010	2.27 ± 0.093	746 ± 14.93
<i>Vitis vinifera</i> cv. Gamay (grapevine)	Cells	Elicited cells with cyclodextrins and methyljasmonate	1.95 ± 0.006	2.43 ± 0.025	937 ± 8.08
		Elicited cells with cyclodextrins and ethanol	1.94 ± 0.015	2.31 ± 0.046	952 ± 9.01
		Cells without eliciting	1.94 ± 0.012	2.32 ± 0.036	878 ± 12.50
<i>Rosa chinensis</i> (rose)	Petals	Young petals	1.95 ± 0.012	2.48 ± 0.025	798 ± 10.40
		Adults petals	1.94 ± 0.015	2.38 ± 0.026	825 ± 12.50

^aResults are expressed as the average of three samples (standard error). ^bOne gram of fresh fruit sample of loquat and quince equals approximately 0.16-0.23 g of lyophilized dry sample. One gram of fresh cells sample of grapevine equals approximately 0.20-0.25 g of lyophilized dry sample. *The percentage (50 and 100%) refers to the size of the fruit at collected time. **At Veraison the % refers to the extent of fruit color change (green to yellow). ***Harvest refers to the optimal state of the fruit for collection. [†]Postharvest refers to the maturation of the fruit on the laboratory at room temperature for several days. ^{††}Ripening refers to the storage of the fruit in the tree for several days. ^{†††}Bruised refers at mechanical damage of fruit in the laboratory and after storage a room temperature for various hours.

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