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Research Paper

Induced parthenogenesis by gamma-irradiated pollen in loquat for haploid production

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Successful haploid induction in loquat (*Eriobotrya japonica* (Thunb.) Lindl.) through in situ-induced parthenogenesis with gamma-ray irradiated pollen has been achieved. Female flowers of cultivar 'Algerie' were pollinated using pollen of cultivars 'Changhong-3', 'Cox' and 'Saval Brasil' irradiated with two doses of gamma rays, 150 and 300 Gy. The fruits were harvested 90, 105 and 120 days after pollination (dap). Four haploid plants were obtained from 'Algerie' pollinated with 300-Gy-treated pollen of 'Saval Brasil' from fruits harvested 105 dap. Haploidy was confirmed by flow cytometry and chromosome count. The haploids showed a very weak development compared to the diploid plants. This result suggests that irradiated pollen can be used to obtain parthenogenetic haploids.

Key Words: *Eriobotrya japonica*, haploidy, gamma-ray irradiation, irradiation dose, ploidy determination.

Introduction

Loquat, *Eriobotrya japonica* (Thunb.) Lindl, is a subtropical evergreen tree, belonging to the tribe *Pyreae*. This non-climacteric fruit, originated in China (Zhang *et al.* 1990), is cultivated between latitudes 20° and 35° North or South (Lin *et al.* 1999). The crop is grown in subtropical areas sharing environmental requirements similar to citrus. Being a minor fruit crop, the breeding activities and genetic studies focused on this species are scarce. To increase knowledge on genetics of loquat requires optimization of biotechnological techniques in this species. Among them, exploring the ploidy manipulation aimed at developing new cultivars is of great interest in woody plants (Ollitrault *et al.* 2008, Ortiz 1998).

Among the different techniques for the alteration of chromosome number, the obtention of haploids has many advantages for the progress of genetic studies, especially in perennial fruit trees that have a long reproductive cycle and high heterozigosity due to self-incompatibility. Haploid and doubled haploid lines are very interesting since they allow to fix traits in homozygous state in a single step. These homozygous individuals are very useful for genome mapping, providing reliable information about the location of major genes and quantitative trait loci for economically important traits (Kush and Virmani 1996). In this sense, haploids have been used for physical mapping (van Leeuwen *et al.* 2003), genetic mapping (Chu *et al.* 2008, Hussain *et al.* 2007, Zhang *et al.* 2008), integration of physical maps (Zhebentyayeva *et al.* 2008) and for the implementation of the reference whole genome sequence in fruit tree species such as citrus (Aleza *et al.* 2009) or peach (Verde *et al.* 2012). Additionally, haploids allow the development of lines that exploit F_1 heterosis, contribute to studies on the process of differentiation of plants and allow studies of totipotency mechanisms in plant cells (Germanà 2009, 2011a, 2011b).

Spontaneous haploid individuals have been identified in several fruit species as apple, pear, peach, plum, apricot, almond, kiwifruit, mango (Crètè 1944, Martínez-Gómez and Gradziel 2003, Sobrinho and Gurgel 1953, Toyama 1974, Zhang *et al.* 2010). However, spontaneous evidence is a rare event, resulting in a limited application; hence artificial haploid induction is necessary for potential use in breeding. Haploid plants can be achieved using several methods: *in vitro* androgenesis (anther-isolated microspore culture) and gynogenesis (ovule-ovary culture), in situ parthenogenesis (pollen irradiation or treatment with chemicals), wide hybridization (chromosome elimination, 'bulbosum' method), selection of twin seedlings, etc. (Germanà and Chiancone 2001). Although androgenesis through *in vitro* anther culture is the most practical method, alternative methods should be evaluated to improve the response.

Induction of maternal haploids embryos by pollination with irradiated pollen has been a effective method in different fruit species as apple (Nicoll *et al.* 1987, Zhang and

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Lespinasse 1991), pear (Bouvier *et al.* 1993, Inoue *et al.* 2004), kiwifruit (Chalak and Legave 1997) and citrus (Aleza *et al.* 2009, Froelicher *et al.* 2007). In the case of loquat, haploidy induction via gynogenesis has not been described. Commonly pollen is irradiated with gamma rays because of their simple application, good penetration, reproducibility, high mutation frequency, and low disposal (lethal) problems (Chahal and Gosal 2002). Gamma-irradiated pollen can germinate on the stigma, grow though the style and reach the embryo sac. Despite, being unable to fertilize the egg cell and the polar nuclei, it stimulates the development of haploid embryos (Musial and Pzrywara 1998). This method requires immature embryo rescue under *in vitro* conditions. Among the different factors influencing the efficiency of the progress, the irradiation dose, the developmental stage of the embryo during *in vitro* culture, the culture media composition and the culture conditions are especially important (Germanà 2011a).

The objective of the present study was to evaluate the response of 'Algerie' loquat to in situ-induced parthenogenesis by pollination with gamma irradiated pollen from 'Changhong-3', 'Cox' and 'Saval Brasil' loquat, followed by *in vitro* embryo rescue. We present results on the viability of irradiated pollen, percent fruit set and the formation of parthenogenetic embryos according to the irradiation doses. Regenerated plants were characterized by DNA flow cytometry.

Materials and Methods

Plant material

The experiments were conducted in 2012 and 2013. Cultivar 'Algerie' was used as female parent, and 'Changhong-3', 'Cox' and 'Saval Brasil' were used as pollen parents. The pollinators were chosen for their intercompatibility (S-allele) and flowering date (**Table 1**). Field experiments were conducted at the Ruchey Cooperative experimental orchards in Callosa d'en Sarrià, Alicante, Spain (latitude: 38°39'5" N, longitude: 0°7'20" W). All plants received standard cultural practices.

Pollen collection, irradiation and pollination

Flower buds were collected just before anthesis. Anthers were excised before pollen dehiscence, and dried at 30°C for 24 h. Dried anthers were crushed and irradiated by gamma-ray radiation to doses of 0, 150 and 300 Gy using a Co-60 source.

Table 1. Loquat accessions used in this study: cultivar, *S*-allele genotype determining intercompatibility and flowering date

Cultivar	*S*-allele genotype	Flowering date[a]
Algerie	*Sb Si*	November 8th (F)
Changhong-3	*Sf*	F –8
Cox	*Sd Sk*	F –10
Saval Brasil	*Sj Sk*	F +2

[a] Flowering date expressed the difference in days from the flowering date of cv. 'Algerie'.

Flowers from 'Algerie' were emasculated before anthesis, hand-pollinated with the different pollen sources and covered with paper bags to avoid contamination by foreign pollen. Control pollinitations with non-irradiated pollen were also performed.

Pollen viability was evaluated in a nutrient medium containing 1.5% sucrose solidified with 0.12% agar, and pH adjusted to 5.7. The media was autoclaved at 120°C for 30 min. For each treatment, five replicates of 100 pollen grains were used. Viability was recorded after 24 h incubation at 26°C.

Seeds extraction and in vitro embryo rescue

Loquat fruits were harvested 90, 105 and 120 days after pollination (dap). Seeds were extracted and surface-sterilized by immersion in a diluted commercial bleach solution (4% w/v sodium hypochlorite) with 0.1% (v/v) of Tween-20® for 10 min, followed by three rinses in sterile distilled water under aseptic conditions for 5, 10 and 15 min.

The sterile seeds were plated on solid medium composed of half-strengh MS (Murashige and Skoog 1962) mineral salts supplemented with glycine 200 $mg.l^{-1}$, thiamine hydrochloride 1 $g.l^{-1}$, nicotinic acid 1 $g.l^{-1}$, pyridoxine hydrochloride 500 $mg.l^{-1}$, ascorbic acid 500 $mg.l^{-1}$, 200 $mg.l^{-1}$ myo-inositol, 20 $g.l^{-1}$ sucrose and 7 $g.l^{-1}$ agar. The pH was adjusted to 5.7 before autoclaving at 120°C for 30 min. Samples were placed in darkness for 3 weeks and then transferred to 16-h photoperiod in a tissue-culture chamber at 24°C ± 2°C.

Acclimatization

After *in vitro* culture, plantlets were transferred to pots (250 cc) containing a mixture of peat and perlite (1:1). The solid medium was carefully washed out of the roots. The potted plantlets were covered with transparent plastic for 7 days in which the cover was gradually removed to allow smooth adaptation of plantlets to lower humidity. Once the plants became acclimatized, they were transferred to a greenhouse at 18–28°C under natural early fall photoperiod.

Flow cytometry analysis and chromosome counting

The plantlets obtained were analyzed by flow cytometry to asses the ploidy level. Leaf samples were chopped in 1.0 ml of nuclei isolation buffer (Partec), filtered through a 50 µm nylon filter (Nybolt, Zürich, Switzerland), and stained with 3.0 ml of coloration solution containing 1 $mg.l^{-1}$ of DAPI (4′-6-diamidino-2-phenylindole.HCl). The fluorescence intensity of the nuclear mixture was measured using a CyFlow® Counter (Partec). Nuclei obtained from 'Algerie' were used as diploid control. The plot data on a semi logarithmic scale resulted in a histogram with peaks from 2C to 100C evenly distributed along the abscissa axis.

Chromosome number was counted in leaf piece cells obtained from *in vitro*-grown haploid plantlet. The leaf sample was pre-treated with 0.04% 8-hydroxyquinoline for 4 h at room temperature (RT) and 3.5 h at 4°C in the dark. Sample

was fixed in 3:1 ethanol-glacial acetic acid for 72 h at RT in darkness, hydrolyzed in 0.25 N HCl for 10 min and washed in distilled water. Fixed leaf piece was placed in digested citrate buffer (0.01 M trisodium citrate-dihydrate + 0.01 M citric acid monohydrate) for 10 min at RT and limited to 2 mm^2. The explant was incubated at 37°C in enzymatic solution (5% cellulase Onozuka R10 + 1% pectolyase Y23) for 20 to 30 min and then immersed in distilled water for 15 min (D'Hont *et al.* 1996). Thereafter a single sample was placed on a slide, the excess of water was removed and 1 drop of fixative solution was added before spread it with fine forceps. Chromosomes slides were colored with 1 $mg.l^{-1}$ DAPI and viewed with a fluorescence microscope.

Results

Pollen germination and fruit set

In 2012, the highest germination rate was obtained using non-irradiated pollen with no significant differences among cultivars, 69.2%, 70.8% and 70.4% for 'Changhong-3', 'Cox' and 'Saval Brasil', respectively (**Table 2**). Germination rate of irradiated pollen decreased significantly compared to non-irradiated pollen (**Table 2**). With the highest irradiation dose (300 Gy) pollen germination capacity of 'Changhong-3' (50.6%) and 'Cox' (42.2%) was significantly reduced as compared with the 150 Gy dose (62.4% and 60.8%, respectively) (**Table 2**).

For all crosses, pollen irradiated at 150 and 300 Gy significantly reduced fruit set compared with the control (**Table 2**). The reduction of the fruit set depended on the male parent and irradiation dose. The highest reduction on fruit set occurred in the cross 'Algerie' × 'Cox' with pollen treated with 300 Gy (**Table 2**).

Influence of fruit-harvest times in embryo rescue

In order to assess the most convenient stage of the embryo evolution for successful embryo rescue, inmature fruits were harvested at 90, 105 and 120 days after pollination (dap), which represented and interval between seed development and fruit enlargement stages (**Fig. 1**).

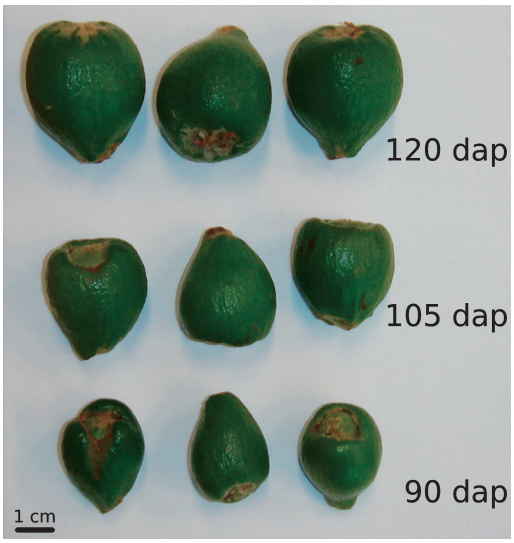

Fig. 1. Fruits harvested 90, 105 and 120 days after pollination (dap), which corresponded to fruit enlargement stages. Bar 1 cm.

In 2012, for all cross combinations and harvest time the highest percentage of seed germination was recorded with non-irradiated pollen. All crosses combinations independently of the harvest time showed the seed germination rates higher using pollen irradiated at 150 Gy than at 300 Gy (**Table 3**). The crosses 'Algerie' × 'Changhong-3' and 'Algerie' × 'Cox', showed a higher percentage of germinated seeds when the fruits were harvested 90 dap with both control and irradiated pollen. For this crosses were no differences in germination rates between fruits harvested 105 and 120 dap. On the other hand, the cross 'Algerie' × 'Saval Brasil' showed no difference in germinated seeds rates beetween fruits harvested 90, 105 and 120 dap using irradiated pollen (**Table 3**).

In 2013 similar trends were observed, though only 300 Gy were applied. The percentage of seed germination was approximately 100% to the three crosses after pollination with non-irradiated pollen (**Table 3**). For 'Algerie' × 'Changhong-3' the frequency of germinated seeds was significantly greater in fruits harvested 90 dap than in 105 and 120 dap using irradiated pollen at 300 Gy. For 'Algerie' × 'Cox' and 'Algerie' × 'Saval Brasil' the percentage of seed germination was lower in fruits collected 105 dap than in 90 and 120 dap using irradiated pollen at 300 Gy (**Table 3**).

Table 2. Mean *in vitro* germination percentages of pollen samples used for in situ-induced parthenogenesis and effect of fruit set in crosses using 'Algerie' as mother plant in 2012

Pollen donor	Irradiation dose (Gy)	Pollen germination[a] ± SE (%)	Flowers pollinated (no.)	Fruit set[b] [no. (%)]
Changhong-3	0	69.2 ± 1.2 a	45	45 (100) a
	150	62.4 ± 1.0 b	400	256 (64.0) c
	300	50.6 ± 1.6 c	400	206 (51.5) d
Cox	0	70.8 ± 1.9 a	45	45 (100.0) a
	150	60.8 ± 0.9 b	400	276 (69.0) b
	300	42.2 ± 0.9 c	400	142 (35.5) e
Saval Brasil	0	70.4 ± 1.0 a	45	45 (100) a
	150	54.4 ± 1.6 c	400	210 (52.5) d
	300	45.2 ± 1.6 c	400	201 (50.3) d

[a] Means of five replicates, 100 pollen grains each. For each cv. percentages followed by the same letters are not significantly different (*Newman and Keuls test, $P = 0.05$; [b] Binomial test).

Ploidy level

A precise estimation of nuclear DNA content of the recovered plantlets was carried out by flow cytometry. A first analysis was made two months after sowing, when the plants developed at least 2–3 true leaves. Individual plants were analyzed, including those obtained from non-irradiated pollen.

Table 3. Effect of pollen irradiation and harvest time (days after pollination) on embryo rescue and parthenogenetic plant production of three loquat crosses in 2012 and 2013 (300 Gy selected as optimal treatment for the assays in 2013)

Cross	dap[a]	Irradiation dose (Gy)	Fruit harvested (no.)		Seed cultured (no.)		Seeds/Fruit (no.)		Germinated seeds [no. (%)][b]		Haploid (no.)		Diploid (no.)	
			2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
Algerie × Changhong-3	90	0	15	10	36	23	2.4	2.3	27 (75.0) a	22 (95.7) b	0	0	27	22
		150	85		177		2.1		80 (45.2) b		0		80	
		300	68	25	181	25	2.7	1.0	52 (28.7) bc	21 (84.0) b	0	0	52	21
	105	0	15	10	34	22	2.3	2.2	26 (76.5) a	22 (100) a	0	0	26	22
		150	85		164		1.9		41 (25.0) c		0		41	
		300	69	40	176	41	2.6	1.0	26 (14.8) d	15 (36.6) c	0	0	26	15
	120	0	15	10	37	22	2.5	2.2	30 (81.1) a	22 (100.0) a	0	0	30	21
		150	86		180		2.1		43 (23.9) c		0		43	
		300	69	38	190	30	2.8	0.8	23 (12.1) d	9 (29.9) c	0	0	23	9
Algerie × Cox	90	0	15	10	33	21	2.2	2.1	28 (84.8) a	21 (100) a	0	0	28	21
		150	92		193		2.1		87 (45.1) b		0		87	
		300	47	24	98	37	2.1	1.5	27 (27.6) c	23 (62.2) c	0	0	27	23
	105	0	15	10	35	18	2.3	1.8	30 (85.7) a	18 (100) a	0	0	30	18
		150	92		199		2.2		40 (20.1) c		0		40	
		300	47	83	107	84	2.3	1.0	25 (23.4) c	20 (23.8) d	0	0	25	2
	120	0	15	10	36	23	2.4	2.3	33 (91.7) a	23 (100) a	0	0	33	23
		150	92		179		1.9		57 (31.8) bc		0		57	
		300	48	105	104	47	2.2	0.4	23 (22.1) c	38 (80.9) b	0	0	23	38
Algerie × Saval Brasil	90	0	15	10	35	19	2.3	1.9	30 (85.7) c	19 (100) a	0	0	30	19
		150	70		142		2.0		42 (29.6) d		0		42	
		300	67	31	135	27	2.0	0.9	23 (17.0) de	14 (51.9) c	0	0	23	14
	105	0	15	10	33	22	2.2	2.2	32 (97.0) a	20 (90.9) b	0	0	32	20
		150	70		133		1.9		25 (18.8) de		0		25	
		300	67	83	144	50	2.1	0.6	12 (8.3) e	10 (20.0) d	3	1	9	9
	120	0	15	10	34	21	2.3	2.1	31 (91.2) bc	21 (100) a	0	0	31	21
		150	70		150		2.1		35 (23.3) de		0		35	
		300	67	97	143	37	2.1	0.4	16 (11.2) e	15 (40.5) c	0	0	16	15

[a] dap: days after pollination.

[b] Different letters indicate statistical differences (Binomial test, $P > 0.05$).

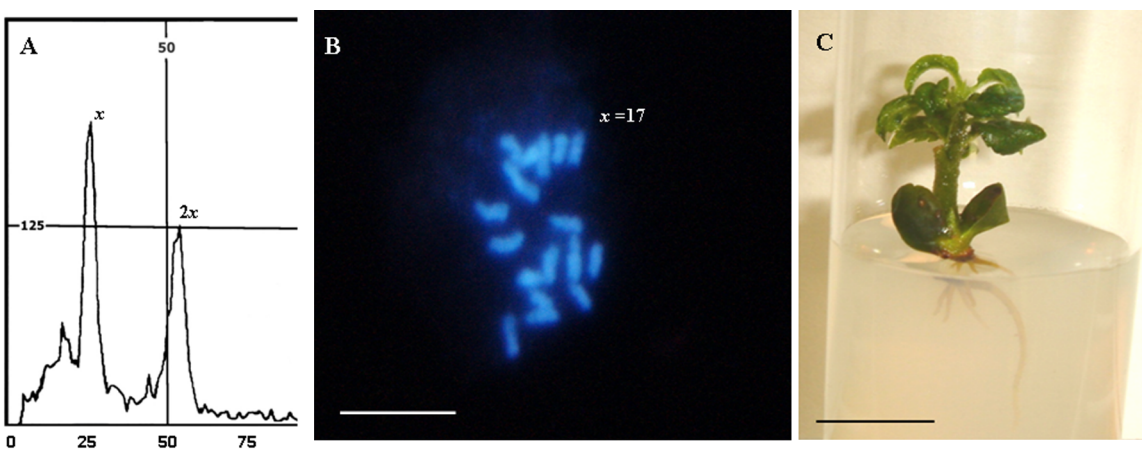

Fig. 2. (A) Flow cytometry histogram of a mixture of tissues from diploid control loquat plant (2x) and the haploid obtained by induced parthenogenesis (x). In the analysis of separate samples, only one peak appeared. Bar 10 μm; (B) Chromosomes count on loquat leaf piece of the haploid cell (2n = x = 17). Bar 10 μm; (C) Haploid plant subcultured in test tube. Bar 1 cm.

In 2012, all the plants produced by non-irradiated pollen or pollen irradiated at 150 Gy were diploid. Three haploid plants were obtained from 'Algerie' pollinated with 300-Gy-treated pollen of 'Saval Brasil' from fruits harvested 105 dap (**Table 3**). However, the plants died during acclimatization due to a toxic phytosanitary spray. No further ploidy analysis was carried out.

In 2013, in the cross 'Algerie' × 'Saval Brasil' embryo rescue at 105 dap resulted in one haploid out of 10 recovered plants. The haploid level was confirmed by flow cytometry and chromosomes count in a leaf sample (17 chromosomes) from the plantlet maintained *in vitro* (**Fig. 2A**,

2B). The haploid plant presented very small leaves and a reduced growth habit when compared with diploid plants (Fig. 2C).

Discussion

Effect of pollen irradiation on pollen viability and fruit set

Irradiation did affect pollen viability for the two doses tested. The effect of the radiation dose on pollen germination rate is a species-dependent parameter. In some species, this effect is limited, as in the case of apple (Zhang and Lespinasse 1991), mandarin (Froelicher *et al.* 2007) and sweet cherry (Höfer and Grafe 2003), while in some others, as European plum, pumpkin, winter squash and walnut, the gamma-ray dose affects significantly the pollen germination (Grouh *et al.* 2011, Kurtar *et al.* 2009, Peixe *et al.* 2000). We have obtained a significant reduction of pollen germination rate in pollen irradiated at 150 and 300 Gy, compared with non-irradiated pollen, being more reduced at higher doses. Results indicated that loquat is more sensitive to ionizing radiation than other fruit trees. Irradiation doses of 500, 700 and 900 Gy did not affect pollen germination of kiwifruit (Chalak and Legave 1997, Musial and Przywara 1998). Irradiated pollen on Meyer lemon at 900 Gy resulted in a germination rate of 78% with no significant differences between the different doses of irradiation applied: 150, 300, 600, 900 Gy (Froelicher *et al.* 2007).

In loquat, fruit set was affected by pollen irradiation in all essayed crosses, the degree to which irradiated pollen affected fruit set was dose-dependent. Similar results were obtained in apple (Zhang and Lespinasse 1991), pear (Bouvier *et al.* 1993), citrus (Froelicher *et al.* 2007), cacao (Falque *et al.* 1992), melon (Lotfi *et al.* 2003) and European plum (Peixe *et al.* 2000). However, its effect on fruit set is not always evidenced, Chalak and Legave (1997) observed in kiwifruit that crosses with pollen irradiated at high doses (700 and 900 Gy) resulted in fruit yield not significantly different from crosses made with control pollen. Hence, the effect of irradiated pollen on fruit set can be explained by the damage caused by low levels of irradiation on the generative nucleus while maintaining its capacity to fertilize the egg cell leading to a successful hybridization (Sestili and Ficcadenti 1996). In citrus, higher doses of irradiation resulted in an increase of small seeds (Ollitrault *et al.* 1996). The small seeds corresponded to empty seeds without embryo. In other species, the irradiation causes a decrease in the number of seeds, mainly due to the aborted seeds. In Arabidopsis the abortion rate at 400 Gy was 50% (Yang *et al.* 2004). In loquat, we obtained a reduction of fruit set varying approximately from 30% when a dose of 150 Gy was used to 50% for a dose of 300 Gy. Besides of reduction of fruit set, pollination with irradiated pollen causes early fruit drop in some species, an explanation could be that the pollen tube cannot reach the egg cell (Grouh *et al.* 2011, Peixe *et al.* 2000).

Induced parthenogenesis

The ratio of haploids per seeds cultured was 3/422 and 1/114 in the first and second experiment respectively, both in the cross 'Algerie' × 'Saval Brasil'. These results are higher than those obtained in pear trees with 2/594 (Bouvier *et al.* 1993) but lower than those on kiwifruit with 18/44 and 21/21 for different doses (Chalak and Legave 1997) and citrus with 1/11 and 1/26 for Clementine and 'Fortune' (Froelicher *et al.* 2007). It should be considered that some of the diploids recovered may be doubled haploids, but this point could not be confirmed, and we have focused on obtaining haploid plants. At this point, we cannot rule out the introgression of pollinator genome fragments after gynogenesis induced by irradiated pollen, but as it has already been demonstrated in different species, such as kiwifruit (Pandey *et al.* 1990), pear (Bouvier *et al.* 1993), and citrus (Aleza *et al.* 2009), this would be rare.

It seems that in order to induce parthenogenesis donor pollen plays a crucial role, as haploids were only recovered when 'Saval Brasil' was used as donor. The effect of the pollen donor genotype on the parthenogenesis efficiency is a fact established by other researchers in other species (Todorova and Ivanov 1999).

The effect of irradiation dosage of pollen for inducing parthenogenesis varies among species. In this experiment, we found that 300 Gy was the optimum dose for recovery haploid embryos, in fact, none haploids were recovered when a lower dose of 150 Gy was used. Doses between 25 and 50 Gy were the best ones for squash (Kurtar *et al.* 2002), 250 Gy for pear (Bouvier *et al.* 1993), 300 Gy for citrus (Froelicher *et al.* 2007), 200 and 500 Gy for apple (Zhang and Lespinasse 1991) and 1.500 Gy for kiwifruit (Chalak and Legave 1997).

It is difficult to find haploid regeneration in loquat following alternative strategies (Germanà 2006). Our group has previously attempted haploidy induction via anther culture, but despite analyzing the effect of different factor only one triploid plant was regenerated via embryogenesis and no haploids were recovered (Blasco *et al.* 2015). Using induced parthenogenesis we have succeeded in the obtention of haploid plants under specific conditions and these results could be confirmed in two years. The recovered haploids showed slow growth and weakness. Many studies with different species have demonstrated that haploid plants have weak growth vigor (Froelicher *et al.* 2007, Riddle *et al.* 2006).

In conclusion, in loquat is possible to induce parthenogenesis by irradiated pollen. This is the first report of successful induction of haploid embryos by in situ parthenogenesis mediated by pollen irradiated in this specie. The experiment allowed the obtention of haploid plants from 'Algerie'. The efficiency of embryo induction in this specie is determined by several factors, such as radiation dose and genotype. All haploids were obtained by irradiation dose of 300 Gy. Results showed the higher efficiency of parthenogenesis induced by irradiated pollen compared with anther culture for production of haploid loquat plants.

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