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Original article

Comparison of two *in vitro* radiation-induced mutagenesis protocols for the Citrus rootstock Swingle citrumelo

Comparación de dos protocolos de radiomutagénesis *in vitro* para el portainjertos cítrico citrumelo Swingle

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ABSTRACT: In order to establish an efficient *in vitro* radiation-induced mutagenesis scheme for the rootstock Swingle citrumelo, two protocols were compared: Protocol 1 (seeds were irradiated and axillary shoots from the regenerated plantlets were propagated) and Protocol 2 (epicotyl segments were irradiated and the induced adventitious shoots were propagated). Radiosensitivity against ^{60}Co gamma rays, shoot induction and three root induction treatments (MS, MS + NAA and MS + IBA) were evaluated. The efficiency regarding the total number of plants and the time required until their acclimatization was compared. The mutated population size was estimated. Mutagenic doses (LD30) of 29 and 37 Gy were determined for seeds and epicotyl segments, respectively. Shoot induction was higher than 93 % in both protocols. Root induction was significantly higher for adventitious shoots on MS + NAA, due to a higher secondary root induction (92 %), which also enhanced their adaptation to substrate (91,8 vs. 70,4 %). Starting from 1000 seeds, 50 and 70 mutants were estimated from Protocols 1 and 2, respectively, considering the proposed LD30 and 0,5 % frequency of induced mutations. It was demonstrated that Protocol 2: radiation-induced mutagenesis based on adventitious organogenesis was more efficient than Protocol 1, based on seed irradiation and axillary shoots propagation; since half the time (seven months) is required to obtain a higher number of mutants. It was mainly due to a high root induction potential and a better root architecture, which allowed for a higher plant *ex vitro* survival.

Key words: induced mutation, radiosensitivity, micropropagation, *Citrus*.

RESUMEN: Con el objetivo de establecer un esquema de radiomutagénesis *in vitro* eficiente para el portainjertos citrumelo Swingle, se compararon el Protocolo 1 (se irradiaron semillas y se propagaron los brotes axilares de las plántulas regeneradas) y el Protocolo 2 (se irradiaron segmentos de epicótilo y se propagaron los brotes adventicios). Se evaluó la radiosensibilidad frente a rayos gamma de ^{60}Co, la brotación y tres tratamientos de enraizamiento (MS, MS+ANA y MS+AIB). Se comparó la eficiencia en cuanto al total de plantas y el tiempo, hasta su adaptación a sustrato y se estimó el tamaño de la población mutada. Se determinaron dosis mutagénicas (DL30) de 29 y 37 Gy para semillas y segmentos de epicótilo, respectivamente. La brotación fue mayor del 93 % para ambos protocolos. El enraizamiento fue significativamente mayor para los brotes adventicios en MS+ANA (0,5 mg L^{-1}), debido al mayor enraizamiento secundario (92 %), que favoreció su adaptación a sustrato (91,8 vs 70,4 %). A partir de 1000 semillas, con las DL30 propuestas y considerando una frecuencia de mutaciones inducidas de 0,5 %, se estimaron 50 y 70 mutantes en los Protocolos 1 y 2, respectivamente. Se demostró que el Protocolo 2 basado en organogénesis adventicia es más eficiente que el Protocolo 1, basado en irradiación de semillas y propagación de brotes axilares; pues permite obtener mayor número de mutantes en la mitad del tiempo (siete meses), debido principalmente a la elevada capacidad de enraizamiento y mejor arquitectura radicular, favoreciendo una mayor supervivencia *ex vitro* de las plantas.

Palabras clave: *Citrus*, micropropagación, mutación inducida, radiosensibilidad.

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INTRODUCTION

Swingle citrumelo (*Citrus paradisi* Macf. x *Poncirus trifoliata* (L). Raf.) is among the most commercially used rootstocks, not only in Cuba (1) but also worldwide (2,3). It is a vigorous tree that induces high yields, is resistant to poor drainage and has moderate drought tolerance. However, like other trifoliate rootstocks, it grows poorly on calcareous soils (4). Thus it is necessary to improve its tolerance to lime-induced iron deficiency; which is a problem for important areas worldwide including Jagüey Grande (Matanzas), an important Cuban citrus-growing region (5). Likewise, it is important to reduce its architecture, since dwarf trees are required in high-density plantings, as a strategy for Huanglongbing management (6).

Radiation-induced mutagenesis has allowed for inducing dwarfism as well as tolerance to different biotic and abiotic stresses in a number plant species (7). In the case of *Citrus*, 15 mutant cultivars have been registered between 1970 and 2017 in the Mutant Variety Database (MVD) of the International Atomic Energy Agency (IAEA) (8). These are mostly, scion cultivars improved for fruit size and quality, seed number reduction, late ripening, yield and disease tolerance (9,10).

For Citrus, particularly *Citrus* rootstocks, traditional sexual hybridization is more limited by their reproductive biology (apomixis), long juvenile periods and high heterozigocity (11). In this sense, *in vitro* radiation-induced mutagenesis is a breeding alternative as it combines the advantages of mutation induction with biotechnological techniques (12). In fact, five *Citrus macrophylla* mutants with higher salt tolerance have been reported, which have been obtained by radiation-induced mutagenesis and *in vitro* selection (13,14).

For *in vitro* mutagenesis approaches in *Citrus*, different explants are used including seeds, buds, calluses, protoplasts, epicotyl segments and shoot tips (15). However, all of them require the establishment of optimal irradiation conditions and efficient propagation protocols (7), as these responses are also genotype and explant dependent (16).

In order to establish an efficient *in vitro* radiation-induced mutagenesis scheme for Swingle citrumelo, two different protocols are compared in this work: in Protocol 1 seeds are irradiated and axillary shoots from the regenerated plantlets are propagated; in Protocol 2 epicotyl segments are irradiated and the induced adventitious shoots are propagated. Radiosensitivity against ^{60}Co gamma rays is evaluated for both explants; percentages of shoot and root induction and substrate adaptation are determined and the efficiency of both protocols is compared regarding the final number of substrate-adapted plants and time required for their adaptation. Based on these results, the size of the mutated population required to obtain an adequate number of putative mutants for each protocol is estimated.

MATERIALS AND METHODS

Plant material

Ripen fruits were collected at the UCTB “Félix Duque” in Jagüey Grande, Tropical Fruit Research Institute (IIFT), from Swingle citrumelo trees that showed 40 % polyembryony and an average of 1,8 plantlets per seed (data not shown).

Explants

Seeds and epicotyl segments were the explants irradiated in Protocols 1 and 2, respectively.

Sees were removed from fresh fruits, de-shelled and disinfested with 0.7 % sodium hypochlorite and transferred to fresh, basal Murashige and Skoog medium (MS) without vitamins, sucrose or plant growth regulators (PGR). After irradiation, seeds were immediately transferred to fresh medium, one seed per culture tube and germinated for 30 days in a controlled growth room (25 °C, 60% relative humidity, 16-h light/8-h darkness photoperiod and illuminance of 2500 lx provided by Chiyoda fluorescent lamps. Germinated plantlets were transferred to liquid MS containing myoinositol (0.1 g L^{-1}) and 30 g L^{-1} sucrose. These were subcultured for 60 days until there were at least three internodes. In polyembryonic seeds, only the most vigorous plantlet per seed was selected.

Epicotyl segments were obtained from etiolated plantlets (regenerated form seeds germinated in darkness on basal MS for 30 days). Segments of approximately 0.5 cm were cut from the epicotyl region and put on Petri dishes containing basal MS. After irradiated, these were immediately transferred to Petri dishes with shoot induction medium: Murashige and Tucker (MT) supplemented with 2.0 mg L^{-1} of 6-benzylaminopurine (6-BAP) (16) and they were kept in controlled growth room, in darkness for shoot induction.

Protocols

A diagram of the evaluated protocols is shown in Figure 1. In Protocol 1, seeds were irradiated and axillary shoots from regenerated plantlets were propagated. In Protocol 2, epicotyl segments were irradiated and the induced adventitious shoots were propagated. Both propagules were subjected to elongation, root induction and substrate adaptation stages.

Radiosensitivity curves

Explants were irradiated at 10, 20, 30, 40 and 50 Gy of Cobalt 60 (^{60}Co) gamma rays, at 35 ºC and a dose rate of 14 Gy/min. The dose range was selected based on previous radiosensitivity studies of these explant types on *Citrus* spp. (16,17). A randomized experimental design was used. One seed per culture tube and 20 seeds per treatment (dose) were irradiated in two independent experiments. For epicotyl segments, two Petri dishes per dose were irradiated in two independent experiments. Each Petri dish contained 15 and 20 segments in the first and second experiment, respectively. The fraction of germinated seeds (GSF), that is, seeds with entire shoots from the total number of seeds and the fraction of responsive segments (RSF), that is, segments with induced adventitious shoots out of the total number of segments, were considered as radiosensitivity criteria for seeds and epicotyl segments, respectively.

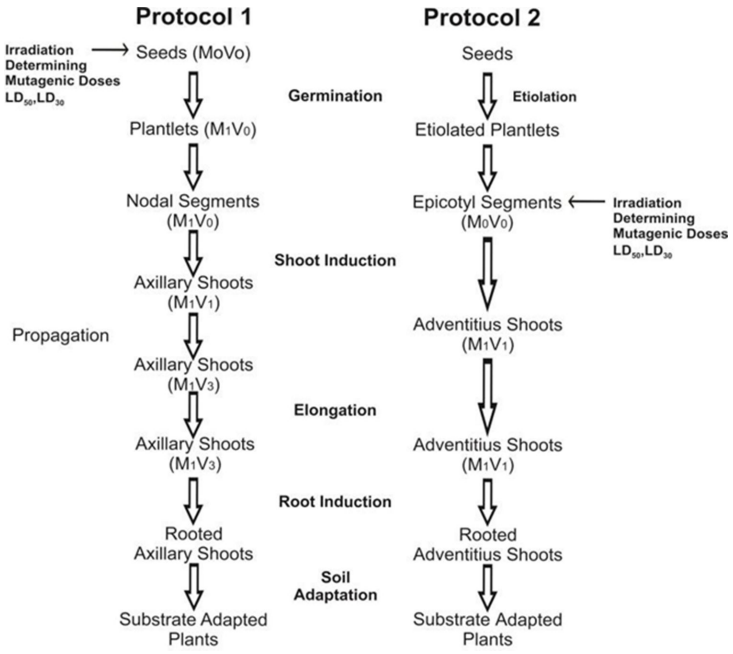


Figure 1. Swingle citrumelo *in vitro* radiation-induced mutagenesis protocols compared in the study.

In vitro propagation

Shoot induction and elongation

For axillary shoot induction, nodal segments were transferred to shoot induction medium consisting of MS supplemented with 6-BAP (1 mg L^{-1}) and 1-naphthalenacetic acid (NAA) (0.1 mg L^{-1}) (18), in controlled growth room for 60 days. To isolate the mutant sectors, axillary shoots were dissected and propagated through two subcultures until M1V3 generation. For adventitious shoot induction, epicotyl segments were kept in shoot induction medium (16), in darkness for 15 days in controlled growth room, the 16-h light/8-h darkness photoperiod was thereafter restored for 60 days. Shoot induction percentage (SI), shoot length (SL) (mm) and number of shoots per explant (SE) were evaluated in 150 explants for each protocol. For SE, only adventitious shoots of approximately 10 mm were recorded, since the shorter ones do not develop further, according to previous observations and other authors' reports (19).

Later, shoots were transferred to elongation medium consisting of MS supplemented with 6-BAP (0.5 mg L^{-1}) and gibberellic acid (GA3) (0.1 mg L^{-1}) (20). Based on the average length of axillary (21) and adventitious shoots (19) of several *Citrus* species, the elongation efficiency (EE) was evaluated as the percentage of shoots longer than 15 mm or 10 mm for axillary or adventitious shoots, respectively. It was determined after 30 culture days in 150 shoots per each protocol.

Root induction and survival in the substrate

Three root induction treatments were compared: I) MS (half strength MS (MS (1/2)) + 30 g L^{-1} sucrose), II) MS + NAA (MS (1/2)) + 30 g L^{-1} sucrose + 0.5 mg L^{-1} NAA) and III) MS + IBA: (MS (1/2) + 30 g L^{-1} sucrose + 0.5 mg L^{-1} 4-3 indolebutyric acid (IBA)). After 60 days, root induction percentage (RI), number of roots per explant (RE) and average root length (RL) were determined. The percentage of secondary root induction (SRI) and number of secondary roots per explant (SRE) were also evaluated.

Rooted shoots were transferred to 1 L plastic containers, with a mixture of soil and vermicompost. Soil was disinfected in an oven at 200 ºC for two hours and mixed with vermicompost (50 %:50 %). Plants were protected with a transparent plastic cover for 15 days. Hand watering was applied three times a week between 8:00 and 10:00 AM until substrate saturation, using MS (1/2) in the first week and running water thereafter. During the third week, the cover was removed one hour daily in the morning.

From the fourth week, when new leaves emerged, the cover was removed for one more hour daily until plants were left completely uncovered. The percentage of survival in the substrate (S) was determined after 30 days.

Data analysis

Dose-effect curves were obtained by fitting radiosensitivity values in Origin-PC. To compare shoot, root induction and substrate adaptation, means, standard deviations and standard errors of every character were calculated Adjustment for normality and variance homogeneity were determined by the Kolmogorov-Smirnov and Levene tests, respectively.

SE and SL values were compared by Mann-Whitney and Student's t-tests, respectively. RL values were analyzed by a one-way ANOVA followed by a Tukey multiple comparison test. For RE values, a Kruskall-Walis followed by a Dunn test were applied (SPSS V19). To compare SI and EE percentages, the confidence intervals were calculated for $p<0.05$. In the case of RI and S percentages, a Chi-square (χ^2) test for proportion comparison was performed (Statgraphics Plus V5.1).

RESULTS AND DISCUSSION

Radiosensitivity and mutagenic doses

From the dose-effect curves, median lethal doses (LD50) of 40 and 45 Gy for seeds and epicotyl segments, respectively were determined (Figure 2).

Although a higher LD50 value (200 Gy) was found for *C. suhuiensis* 'limau madu' seeds desiccated to 25.5 % moisture content before irradiation (17), the same authors reported a LD50 of 50 Gy when *C. suhuiensis* 'limau langkat' seeds were irradiated without desiccation. This value is closer to our result, where seeds were irradiated immediately after being removed from fresh fruits, with high moisture content.

This could cause the differences among the results, since oxygen and water content are the main factors modifying radiosensitivity in seeds (22,23).

For epicotyl segments, lower LD50 values were reported for other *Citrus* species such as Fremont and Thomas mandarins, Murcott tangor and Rangpur lime, which varied between 22 and 34.5 Gy (16). Although a genotype-dependent response may explain these differences, other factors such as culture and irradiation conditions (temperature, dose rate, gamma radiation energy) as well as physiological status of plant material also contribute to the differences.

Mutagenic dose selection depends on the breeder's experience and on the plant material genetics and physiology. Frequently, the use of the LD50 (±10 %) is recommended as it is considered the dose at which the highest frequency of mutations occurs (23,24). However, many deleterious mutations occur which cause the loss of 50 % of the irradiated material. Therefore, many studies recommend an intermediate dose to allow for inducing mutations without a high level of physiological damage; for instance, the dose that reduces by 30 % the survival (LD30) or growth (GR30) of the plant or propagule (16,23,25). In this study, LD30 of 29 Gy and 37 Gy were determined and recommended in Protocols 1 and 2, respectively.

Shoot induction and elongation

High shoot induction percentages and similar SE values were obtained for both axillary and adventitious shoots (Table 1).

For axillary shoots, the 2.16 SE value is higher than those obtained in previous studies using the same 6-BAP concentration (1 mg L^{-1}) either for the same genotype (SE=1,14) (26), as well as for Cleopatra mandarin, sour orange and *C. macrophylla* (1 to 1.5) (21).

The SE value (2.01) for adventitious shoots was lower than those reported for this explant type in scion cultivars (10), while it was higher than those reported for other rootstocks such as Cleopatra mandarin, sour orange and *C. macrophylla* (SE 0.8 - 1.4) (27). For Citrus species, a marked genotype effect on either the regeneration pathway or environment and hormone requirements for optimal formation of the adventitious shoot has been demonstrated (27). Thus, these conditions should be well established for each cultivar.

The stimulating effect of 6-BAP and its interaction with other PGRs on direct or indirect organogenesis in different species has been demonstrated (19,21). In this study, 2 mg L^{-1} of 6-BAP allowed for adventitious shoot induction, the same concentration previously used for several Citrus cultivars (16), while some authors used up to 6 mg L^{-1} for genetic transformation of citrange Carrizo adventitious shoots (28). However, other researchers agree with the fact that 6-BAP concentrations higher than 1 mg L^{-1} resulted in shoot induction inhibition (18-20). Therefore, in further studies to increase the multiplication rate of adventitious shoots, 6-BAP concentrations should be carefully evaluated as well as its combination with other PGRs, considering their synergistic effect.

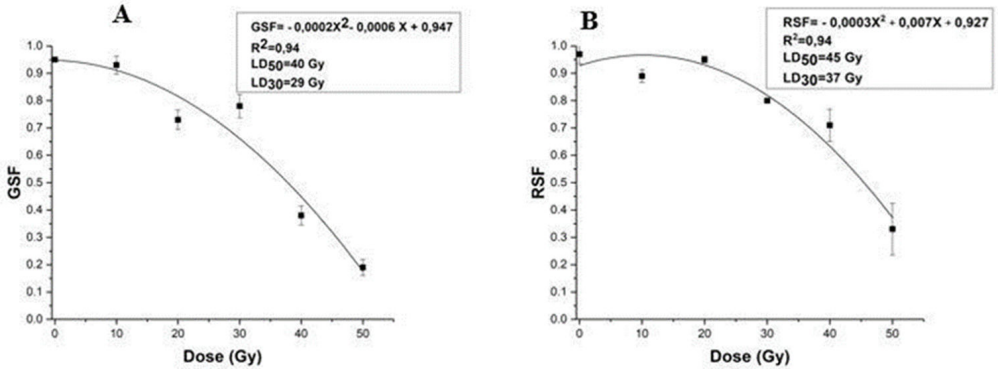


Figure 2. Dose-effect curves against ^{60}Co gamma rays for seeds (A) and epicotyl segments (B) of the rootstock Swingle citrumelo.

Table 1. Induction and elongation of axillary and adventitious shoots of Swingle citrumelo

		Axillary shoots	Adventitious shoots	
Shoot induction	SE	2,16 ± 0,07	2,01 ± 0,07	n.s. p<0,01
	SI (%)	94,6	93,5	n.s. p<0,05
Elongation	SL (mm)	26,5 ± 0,8	15,1 ± 0,5	** p<0,01
	EE (%)	94,3	95,3	n.s. p<0,05

SE and SL expressed as mean ± standard error

A good response of both propagules to the elongation medium was observed. After 30 days, 94.3 % of axillary shoots exceeded 15 mm (SL = 26.5 mm) which is almost three times their initial length and 95.3 % of adventitious shoots was longer than 10 mm (SL = 15.1 mm) (Table 1).

Root induction and survival in the substrate

After two months, there were no significant differences between root induction of axillary shoots on basal MS (67 %) and on MS supplemented with NAA or IBA (69 %) (Figure 3).

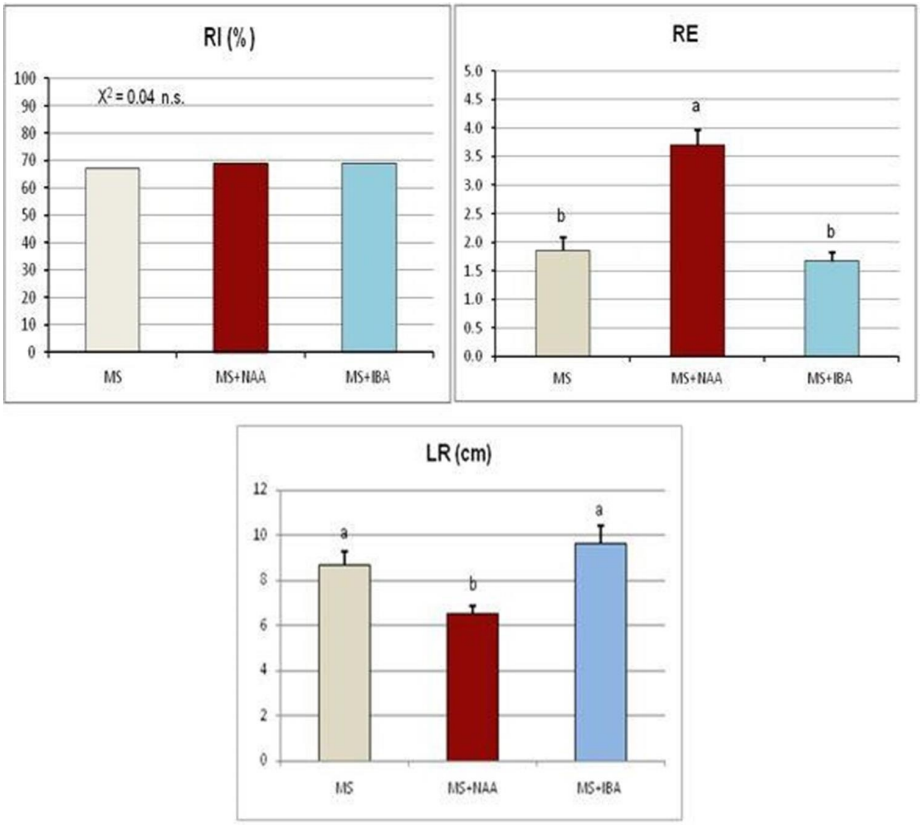
In previous results, 65 and 95 % of root induction in Cleopatra mandarin and sour orange were obtained, respectively (21) with 2 mg L^{-1} of NAA; while 80 % of rooting was induced for *C. macrophylla* using a combination of AIB (1 mg L^{-1}) + indolacetic acid (IAA) (1 mg L^{-1}). On the contrary, our results suggest certain competence of Swingle citrumelo axillary shoots to induce rooting, independent of exogenous PGRs. In fact, some authors found up to 55 % root induction for axillary shoots of this rootstock in RMAN (18): a standard rooting medium for *Citrus*, with a very low NAA concentration (0.02 mg L^{-1}). Further studies including endogenous auxins evaluation in Swingle citrumelo axillary shoots would be necessary in order to corroborate this hypothesis.

Although auxin addenda did not increase root induction percentage for axillary shoots compared to basal MS, significant differences among treatments were found regarding number of roots per explant (RE) and average root length (RL). IBA (0.5 mg L^{-1}) induced longer roots (9.6 cm) compared to NAA-supplemented medium (6.5 cm). However, the last one induced a significantly higher number of roots per explant (3.7) compared to basal MS (1.7) or MS+IBA (1.7).

The highest root induction and 5,66 RE average was previously found for Swingle citrumelo (26) when NAA (2 mg L^{-1}) was used When explants from woody species are propagated *in vitro*, it is important to develop enough roots to allow a successful transplanting and *ex vitro* survival. Therefore, for axillary shoots, a better rooting was obtained with NAA (0.5 mg L^{-1}) since it induced a higher number of roots per explants while the average root length was not significantly different from that obtained with basal MS.

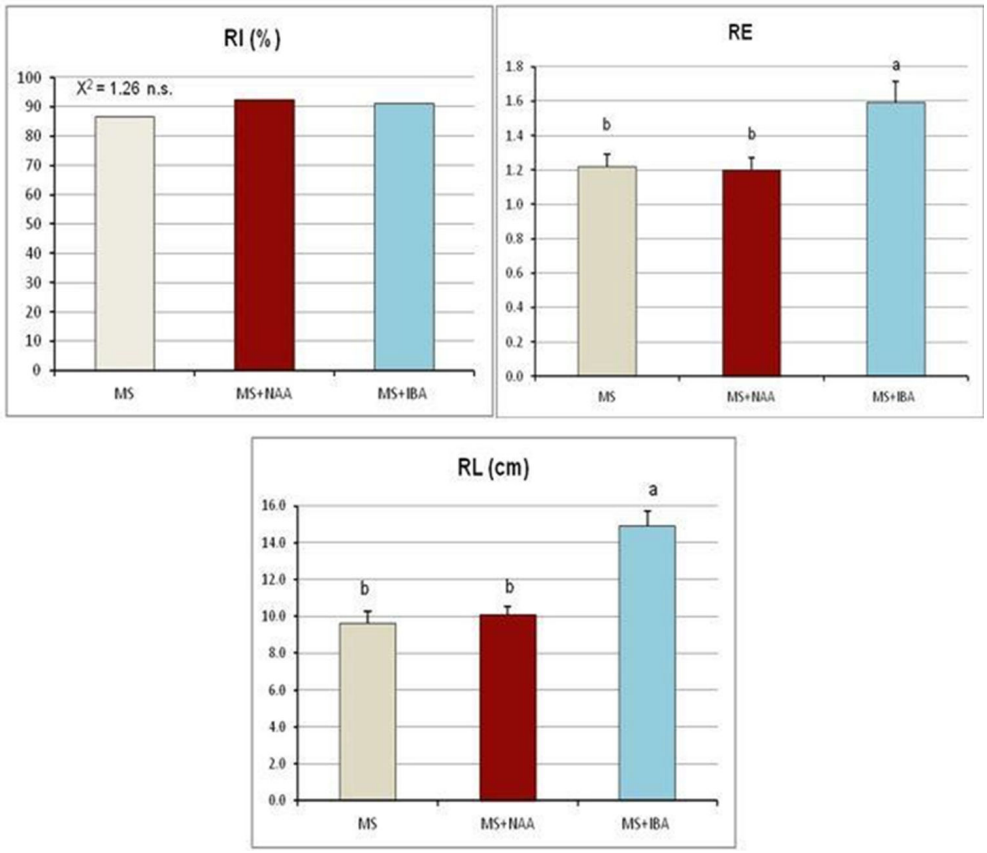
Adventitious shoots showed high rooting percentages, even on medium without PGRs. Their rooting capacity was higher than that of axillary shoots: 86 % RI in basal MS, 93 % in MS + NAA and 91 % in MS + IBA (Figure 4).

In this study, a differential response to root induction between axillary and adventitious shoots was observed. Different mechanisms regulating biosynthesis, transport or response to auxin signals could be acting in each explant type. For instance, it is known that the TIR1/AFB-type auxin receptors are part of a ligase ubiquitin-protein complex ($SCF^{TIR1/AFB}$). Also, there are proteins (Aux/IAA) which act as repressors of the auxin response transcription factors (*ARF*).



Different letters indicate significant differences (p<0.05). RI (%) were not significantly different (X^2, p<0.05)

Figura 3. Root induction percentage (RI), roots per explants (RE) and average root length (RL) for axillary shoots after 60 days on MS, MS + NAA (0.5 mg L^{-1}) and MS+IBA (0.5 mg L^{-1}).



Different letters indicate significant differences ($p<0.05$). RI (%) were not different significantly (X^2, $p<0.1$)

Figure 4. Root induction percentage (RI), roots per explants (RE) and average root length (RL) for adventitious shoots after 60 days in MS, MS+NAA (0.5 mg L^{-1}) and MS+IBA (0.5 mg L^{-1}).

When auxins are perceived intracellularly, the $SCF^{TIR1/AFB}$ complex targets the Aux/IAAs proteins for degradation. When these are removed, the *ARF* factors become active, which on their turn activate the auxin responses. Only in *A. thaliana*, 6 *AFBs*, 29 AUX/IAAs and 23 *ARFs* are known and their combination can result in different transcriptional responses depending on their presence in certain tissues or the plant physiological status. Even more, the differential affinity of these receptors for synthetic analogues of auxin makes the response signaling pathways much more complex (29).

For adventitious shoots, primary roots were more (1.6) and longer (14.9 cm) in MS + IBA (Figure 4), in contrast with other studies in *Citrus* spp. that report better root induction using NAA than using IBA or IAA (21,30). However, form 30 days, secondary roots induction was observed in NAA supplemented medium, which became significantly higher (92 %) at 60 days (Figure 5). In this culture medium the induction and elongation of primary roots was replaced by secondary roots emission and the highest average of secondary roots per explant was obtained.

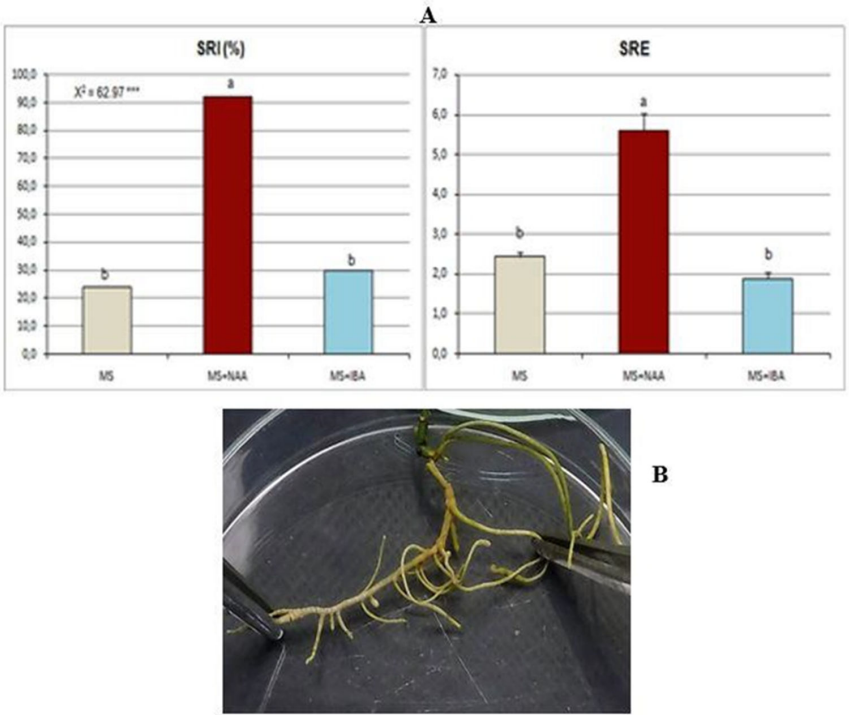
At 60 days, axillary shoots showed lower secondary root induction than adventitious shoots: 10, 10 and 8 % for basal MS, MS + NAA and MS + IBA, respectively, with no statistical differences among them.

Exogenous auxin action in the formation of a complete root system is well known in several species. In fact, NAA is associated to this process in maize plantlets. It was demonstrated that low NAA concentrations (0.002 mg L^{-1}) inhibit primary root elongation thus increasing lateral roots emission by a mechanism associated to the pericycle cells length (31).

Ex vitro survival percentages were compared based on the previous rooting treatment (Table 2). No significant differences were seen for axillary shoots survival, regardless their previous rooting treatment. On the other hand, adventitious shoots from MS + NAA showed the highest survival (91.8 %), probably due to a more efficient root system, involving secondary roots development. Except this, the remaining treatments ranged only between 58 and 74 % of survival. Thus, alternative acclimatization conditions should be proven, including substrate mixtures, fertilization, illumination as well as fungicide application before transfer to substrate.

Efficiency comparison

Fifteen plants survived per each germinated plantlet with Protocol 1, while 19 plants per initial plantlet are produced with Protocol 2 (Table 3).



Different letters indicate significant differences ($p<0.05$), X^2 test for SRI ($p<0.01$)

Figure 5. (A) Secondary shoot induction percentage (SRI) and secondary roots per explant (SRE) for adventitious shoots, after 60 days on MS, MS + NAA (0.5 mg L^{-1}) and MS + IBA (0.5 mg L^{-1}). (B) Secondary roots developed by an adventitious shoot 60 days in MS + NAA (0.5 mg L^{-1}).

Table 2. Percentage of *ex vitro* survival (S) of axillary and adventitious shoots based on their previous root induction treatment.

Root induction treatment	Axillary shoots		Adventitious shoots	
	N	S (%)	N	S (%)
MS	29	58,6	50	68,0 b
MS+NAA	27	70,4	49	91,8 a
MS+IBA	27	63,0	50	74,0 b
χ^2 test		0,86 ns		8,77**

Table 3. Comparison of protocols regarding total number of plants surviving *ex vitro* from each germinated plantlet.

	Protocol 1	Protocol 2
Plants germinated per seed *	1	1
(Nodal/epicotyl) segments per plant	3-4	10-12
Axillary/adventitious shoots induced	6-8	20-24
Axillary shoots propagated (M1V3.)	24-32	-
Elongated shoots [(a)]	30	23
Shoots that induced roots [(b)]	21	21
Ex vitro survival [(c)]	15	19

[(a)] EE 0.94 and 0.95 EE for axillary and adventitious shoots, respectively
[(b)] 69 and 93 % RI for axillary and adventitious shoots, respectively (0.5 mg L^{-1} NAA)
[(c)] 70 and 92 % S for axillary and adventitious shoots, respectively

As seen in the table, Protocol 1 includes a propagation step of axillary shoots until M1V3 generation in order to isolate mutant sectors and reduce chimerism. In vegetatively propagated tissues which do not undergo a haploid stage (meiotic), the mutated cell most face a diplontic selection. That is, a competition with the remaining diploid, non-mutant cell lineages which generally have a better chance to divide successfully. That is why many recessive mutations are lost (32). In axillary meristems, if the mutant cell divides to certain extent, sectorial chimeras will develop and these axillary shoots will give rise to unwanted chimeric plants. On the contrary, by vegetatively propagating the M1V1 shoots, new axillary buds will probably generate from the mutated sector with a lower chimerism rate. It has been proved that a reduction in 60 - 80 % of citochimeras is achieved by propagating until M1V3 generation (33).

Therefore, Protocol 1 includes the vegetative generation advance to M1V3. By this, the initial value of 2.16 axillary shoots per explant is increased to 32 shoots per germinated seed at the end of the propagation stage. However, it makes Protocol 1 four months longer than Protocol 2 (Figure 6). On the contrary, if the *in vitro* method involves a single-cell stage as in the case of adventitious shoots, chimeras are avoided (15) and the four-month vegetative propagation step is unnecessary. Adventitious shoots also showed a high capacity of secondary rooting and a higher survival in substrate. Because of that, less adventitious shoots were lost during this stage, in comparison to axillary shoots. These results indicate a higher efficiency of Protocol 2 for Swingle citrumelo.

Mutated population size

By increasing the number of treated explants one may expect a higher probability of success; that is, obtaining the desired mutants depends on the mutated population size. To estimate this size, the number of vegetative propagation cycles after irradiation, root induction and plant survival percentages must be taken into account (33). Considering our results, the number of putative mutants that can be obtained for each protocol can be estimated.

In Protocol 1, starting from a 1000 seeds population (irradiated at LD30), 700 seedlings and 2800 nodal segments will be regenerated, which will yield around 21000 axillary shoots after micropropagation. Considering the rooting and *ex vitro* survival percentages, approximately 10100 plants will be obtained. With Protocol 2, 1000 seeds will produce 12000 epicotyl segments. By irradiating them at LD30, 16800 adventitious shots will be regenerated. After propagation, root induction and substrate survival, approximately 13655 plants will be obtained. Considering that a common induced mutation frequency is in the order of 0.5 percent (33), about 50 and 70 mutants out of 1000 initial seeds can be expected in Protocols 1 and 2, respectively.

Polyembryony rate can affect the total number of plants obtained in these protocols. As most citrus rootstocks, Swingle citrumelo is a polyembryonic cultivar, in which more than one embryo can develop from a single seed. A seed lot with a high polyembryony rate could produce a higher number of seedlings and therefore, putative mutants. Besides, nucellar embryos are more vigorous and competitive than zygotic embryos produced by self-pollination, thus the higher the polyembryony rate, the greater the frequency of nucellar seedlings, with the same mother plant genotype (34). As induced mutagenesis is aimed at improving certain traits without changing the cultivars genetic background, using fruits from greenhouses or controlled field conditions, where the chances of nucellar seedlings are higher, is recommended. On the contrary, the number of hybrid seedlings increases in seeds from open-pollinated fruits as these zygotic embryos are more vigorous. Pollination quality, nutrition and environment among other factors (24) can affect polyembryony rate. Therefore, although the Swingle citrumelo lots in this work showed 40 % polyembryony with an average of 1.8 seedlings per seed (data not shown), only one (the most vigorous) seedling per seed was considered in our analysis (Table 3). In any case, it is recommended to confirm the nucellar origin of the selected mutants.

Somatic embryogenesis has been proposed as a proper approach for genetic transformation and *in vitro* mutagenesis programs in *Citrus* spp. as it offers an important source of unicellular explants (30). However, in spite that high embryogenesis and regeneration rates are obtained, the overall process can be time consuming and survival to transplantation may decrease from 30 to 40 % (35,36). Moreover, Swingle citrumelo ovular explants are recalcitrant to somatic embryogenesis while this genotype is highly responsive to in vitro micropropagation via organogenesis (30). The combination of *in vitro* radiation-induced mutagenesis with adventitious organogenesis proposed in this work, allowed high root induction and survival in seven months. This approach could be considered an alternative to somatic embryogenesis for mutation breeding of this rootstock.

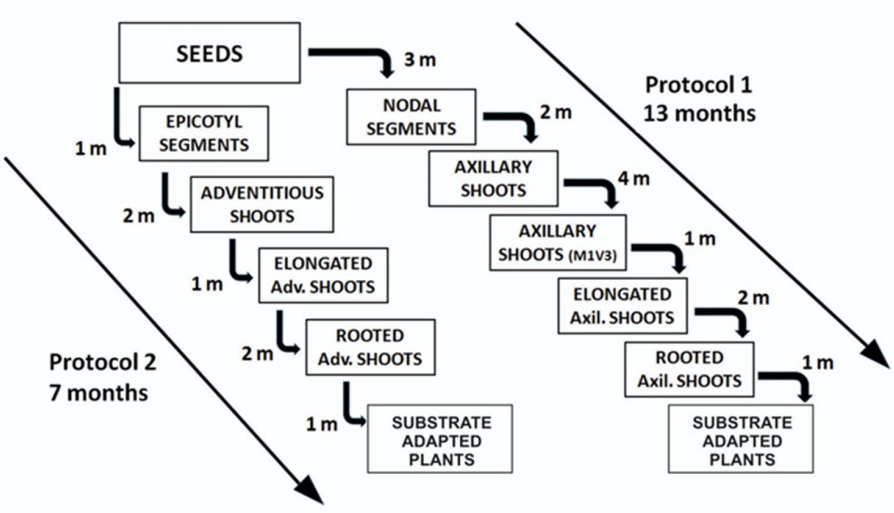


Figure 6. Time frame of the protocols 1 y 2 until *ex vitro* survival of Swingle citrumelo plants.

CONCLUSIONS

For Swingle citrumelo, the *in vitro* radiation-induced mutagenesis protocol based on adventitious organogenesis from epicotyl segments is more efficient than the one based on seed irradiation and axillary shoots propagation, since about half the time (seven months) is required to obtain a higher number mutants. This is mainly due to the higher root induction and a better root architecture, which favor a higher *ex vitro* plant survival.

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