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Full Length Article

Optimization of *In Vitro* Embryo Rescue for Mandarins Triploids (*Citrus reticulata*) Obtained from Diploids Crosses

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Abstract

Triploidy represents a promising method for developing new citrus varieties with seedless fruits. Thus, the objective of this study was to investigate the influence of seed shape size and female parent genotypes factors on the efficiency of embryo rescue technique for the regeneration of triploids seedless hybrids from mandarin's diploids crosses. The culture of immature embryos was initiated from the excised embryos of the four varieties of mandarin (*Murcott honney*, *Nadorcott*, variant of *Murcott* M104 and *Ortanique*). Seeds extracted from ripe fruits and immature embryos derived from abnormal seeds (small and flat) were cultured *in vitro* on Murachige and Tucker (MT) culture medium, supplemented with 1mg/L of acid gibberellic (GA3). The triploid seedlings were identified by the flow cytometry technique. The variance analysis showed that there are significant differences between the varieties studied for all the criteria except for the germination interval having an average value of 3 days. The germination percentage of immature embryos varied from 77% (*Ortanique*) to 92% (*Nadorcott*). Similarly, the aerial growth rate of vitroplants oscillated from 1 mm/day (*Murcott Honney*) to 3.70 mm/day (*Ortanique*) while the root growth rate evolved from 1.90 mm/day (variant of *Murcott* M104) to 3.80 mm /day *(Ortanique)*. In addition, the triploidy rate evolved from 2.70% (*Murcott Honney*) to 13.51% (*Ortanique*). Flow cytometry Analysis revealed that the majority of triploids come from abnormally developed seeds (flat and small), specifically small seeds. This gave a high number of triploid seedlings, and from this the variety *Ortanique* showed the highest rate of regeneration of the triploid seedlings (15.38%) in comparison with the other varieties. The variation in germination rate, seedling growth and triploidy of the citrus varieties studied depends on the genotype and seed shape factors. The development and mastery of this method of preferential selection of triploids opens the way to the creation of populations of varieties of mandarin trees with seedless and easy-to-peel fruits.

Keywords: Citrus; Mandarin; Triploidy; Embryo rescue; Flow cytometry

Introduction

Citrus is a major fruit crop that is widely grown in tropical and subtropical regions around the world. They account for an annual production of over 124.3 million tones worldwide and around 18% of total global fruit production (Mahato *et al.* 2020). Morocco is among the major exporting countries of small-fruited citrus in the Mediterranean region. In a very competitive fresh fruit market, mandarins and clementines are today one of the most dynamic sectors of citrus production and world trade. Mandarins and their diploid hybrids, on the other hand, produce fruits with a lot of seeds, and their planting near clementine orchards can result in a lot of seeded clementines. One of the best strategies to meet the constraints set out above would be to create new triploid varieties of mandarin (2n=3x=27) characterized by sterility (Aleza *et al.* 2010b; Handaji *et al.* 2020; Lourkisti *et al.* 2021a). The triploidy has proved to be a powerful approach breeding programs, particularly in citrus, since seedlessness is one of the greatest consumer expectations (Lourkisti *et al.* 2021b). Moreover, Triploidy has the potential to play a significant role in the coming decades through enhancing biomass and abiotic stress tolerance, both of which have commercial implications (Costa *et al*. 2019; Lourkisti *et al.* 2020). On the other hand, triploid embryos are mostly found in small seeds, between one-third and one-sixth larger than normal seeds, which in most cases, do not germinate in a conventional greenhouse.

To obtain high germination rates, embryo rescue from these small seeds is required (Navarro *et al.* 2002; Aleza *et al.* 2010b). Thus, the rescue of immature embryos *in vitro* with an evaluation of ploidy by flow cytometry have been

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revealed as two decisive methods in the development of effective programs for the selection of triploid plants (Ollitrault *et al.* 1996b; Navarro *et al.* 2003; Aleza *et al*. 2010b; Dalel *et al.* 2020). In addition to seedlessness, embryo rescue has been applied to breeding programs for early ripening citrus (Ramming *et al.* 1990), triploidy or interspecific crosses among other fruit crops such as apple (Druart *et al.* 2000), grape (Angelica *et al.* 2022), banana (Uma *et al.* 2011), mango (Krishna and Singh 2007), persimmon (Hu *et al.* 2013), and peach (Yamada and Tao 2007). As a result of continuous efforts to optimize the *in vitro* rescue technology, the number of obtained spontaneous triploids is insufficient to support improvement programs of seedless plants as protocol efficiency is greatly influenced by several endogenous and exogenous factors: Parental crossing genotypes (Handaji *et al.* 2005; Essalhi *et al.* 2020 and 2021), culture medium composition (Ennaciri *et al.* 2020; Hmimidi *et al.* 2020), plantlet acclimation conditions and the addition of growth regulators are two of the most critical elements impacting rescue efficiency (Mahmoudi *et al.* 2019 and 2020).

Diploid species hybridization from low-frequency divalent gamete fusion or intra-and inter-ploidy crossings can result in the production of new constant triploid hybrids. However, without a highly effective aseptic approach and ploidy event, considerable breeding work based on small F1 hybrid seeds created is unfeasible. In this study, natural hybrids were recovered from nonembryogenic diploid, open-pollinated mandarins using *in vitro* embryo culture. The purpose of this study was to see how the size of the seeds and the genotypes of the female parents affected the efficiency of the embryo rescue method for the regeneration of triploid seedless hybrids from mandarin diploid crossings.

Materials and Methods

Plant materials

Four varieties of mandarin were open pollinated in non-block area during the anthesis on Marsh and April: ***1***. *Nadorcott,* ***2.*** *Murcott honney,* ***3.*** *Ortanique* and ***4.*** variant of *Murcott Honney (*M104*)*. These varieties were planted in the field of the National Institute of Agronomic Research (INRA Kenitra/El Menzeh).

Methods

***In vitro* culture of immature embryos:** At maturity stage, the fruits of the four mandarin's varieties were harvested. Then, after extraction of the seeds, two categories were identified: normal (fully developed) and abnormal seeds (partially developed). The last ones are classified as flat and small (Fig. 1; Table 1). Seeds were classified by size and developmental stage. Size was measured and developmental stage was evaluated by morphological parameters. Perfect seeds (PS) were normal in appearance, totally filled out, and without any malformation. Undeveloped seeds (US) had incomplete development, not totally filled out, wrinkled, and with split outer integument (Fig. 2). Under a laminar flow hood, seeds were surface sterilized for 5 min in a solution of 70% ethanol, followed by immersion for 10 min in 10% sodium hypochlorite. Thereafter, they were cleaned three times with sterile distilled water.

The embryos should be treated very carefully while decortications of the seeds, in order to avoiding damage them. Under aseptic conditions, they were cultured on Murashige and Tucker (1969) culture medium with 1 mg/L of gibberellic acid (GA3), which contained 50 g/L sucrose, 500 mg/L malt extract and vitamins (100 mg/L myo-inositol, 1 mg/L pyridoxine hydrochloride, 1 mg/L nicotinic acid, 0.2 mg/L thiamine hydrochloride, 4 mg/L glycine), and 8 g/L Bacto agar (MT culture media is adjusted to a pH of 5.7 and sterilized at 120°C for 20 min). Baskets cultures are placed in an incubator under 16/18 h (light/dark) photoperiod with light intensity of 1000 lux provided through neon and a temperature of 27°C during the day and 19°C at night. Embryo germination was conducted for 12 weeks in a growth chamber under a 16-h photoperiod with a light intensity of 1000 lux provided by a cool-white-fluorescent lamp, at a temperature of 23±2°C. Embryos showing newly formed cotyledonal leaves were observed and recorded regularly. The percentage of embryo germination was calculated as the number of germinated embryos in the total number of formed embryos. Plant growth allows determining the rate of regeneration. The triploidy rate is calculated. Also, the formulas of all parameters were detailed by (Ennaciri *et al.* 2020).

Ploidy determination by the flow cytometry: Ploidy levels of seedlings were evaluated by flow cytometry using a Partec II cytometer. Nuclei suspensions were made from mature leaf samples and whole seeds. About 0.5 g of leaf tissue was collected from fully or near fully expanded leaves of seedlings two or three months after germination. In the presence of a leaf portion of a triploid control, the tissue was placed in a 60×15 mm polystyrene dish with 0.5 mL of phosphate-buffered saline (PBS) buffer, dithiothreitol 1 mg/L and 0.1% Triton X 100, chopped to a fine mash with a single-edge razor blade. The nuclei were then filtered through a 50-µm nylon filter and stained with 2 mL of 4,6-diamine-2-phenylindole (DAPI) solution (High Resolution DNA Kit Type P, solution B; Partec). The amount of DNA was evaluated by the intensity of the fluorescence re-emitted by the nuclei under UV excitation (365 nm). After a 2-min incubation period, stained samples were run in a Ploidy Analyzer (Partec, PA) flow cytometer. Histograms were analyzed using the dpac v 2.18 software (Partec), which determines peak position, coefficient of variation (CV), and the relative ploidy index of the samples. The triploid control used in this study is Moroccan cultivar "*HANA".*

Statistical data analysis

Quantitative data were analyzed using SAS (Statistical Analysis System version 9.1 and version 5.5) and were subjected to analysis of variance (ANOVA). Means were compared using Duncan's test at a 5% level of significance. Several analyses of variance were performed to compare the means of the variables.

Results

Seed germination and seedling formation

Variance analysis showed a significant effect between the four mandarin varieties for majority of the traits (germination rate and interval, stem and root growth rate) (Fig. 3; Tables 2, 3).

Germination rate and interval: The germination rate varied from 77 to 92% with an average of 83.58%. Three statistically differentiated groups were identified. The first group included the varieties *Nadorcott* and the variant of *Murcott* M104: the second group ***ab*** contained the variety *Murcott Honney* and the group ***b*** with the variety *Ortanique*. For the germination interval, it appears that there is no significant difference between all the varieties studied. The germination was taken approximate 3 days.

Stem growth rate: The aerial growth rate varied statistically according to the genotypes studied (Table 3). It oscillated from 1.00 mm/day to 3.70 mm/day with an average of 2.03 mm/day and from 0.60 mm/day to 1.80 mm/day with an average of 1.10 mm/day during the second and third weeks respectively. Indeed, the growth rate of the stem decreased from the third week for all the varieties studied. This could be explained by the exhaustion of the culture medium used. Similarly, the variety *Ortanique* is relatively characterized by a better stem growth rate with an average of 3.70 mm/day followed by the variant M104. A good development of the foliar system is thus observed for all the regenerated green seedlings.

Root growth rate: The statistical analyzes revealed a significant difference between the four varieties of mandarins for root growth. Thus, three different groups have been highlighted, group ***b*** includes the two varieties *Ortanique* and *Nadorcott*, group ***c*** represents the variant of *Murcott* M104 and group ***bc*** contains the variety *Murcott honney*. In addition, the average value of root growth speed varied from 3.03 mm/day (2^{nd} week) to 2.33 mm/day (3rd week). In addition, a decrease in the evolution of growth took place during the following weeks for all varieties with the exception of the variant of *Murcott* M104.

The Influence of seed size on the parameters studied

Germination rate and interval: There was a significant difference between the four varieties of mandarin in germination rate and seed shape (Fig. 4). The germination

Table 1: Average length, normal, flat and small seeds of each studied variety

Mandarins varieties	Length means of seeds en mm²		
		Abnormally developed	
	Normal	Small	Flat
Ortanique	122.15	23.9	70.4
Murcott honney	116.25	26.2	67.1
Nadorcott	122.2	25.2	55.8
Variant of Murcott (M104)	105	22.15	61.2
Mean (mm²)	116.40 (± 8.10)	24.36 (± 1.75)	63.63 (± 6.46)

Table 2: Percentage and interval germination of seeds from mandarin's varieties

Mandarins varieties	Germination rate (%)	Germination Interval (days)
Ortanique	76.66b	3.00a
Nadorcott	91.85a	2.77a
Variant of Murcott M104	89.28a	2.78a
Murcott Honney	83.73ab	3.00a
Mean	83.58 (± 6.73)	2.88 (± 0.13)

Duncan's test according to a level of significance (5%)

Fig. 1: Mandarin's varieties with their seeds extracted from ripe fruits and classified according to theirs shapes and sizes (a: abnormal, b: normal).

Fig. 2: Comparison of seeds Size, with A: Normal; B: Flat and C: Small seeds

rate was 100% for the normal seeds, but it varied from 55 to 98% for the small seeds and from 66 to 100% for the one with the flat shape. For the variety *Ortanique*, there are two statistically different groups, the first group ***a*** includes the normal and flat seeds which are characterized by a very high germination rate followed by the second group ***b*** which includes only the small form. For *Nadorcott*, the normal seeds forming group ***a*** gave a higher germination rate than the small and flat seeds ***ab***. In the majority of citrus varieties, the germination rate of normal seeds is higher than that of abnormal seeds (flat and small). For germination rate of *Murcott Honney* varieties and its variant M104, normal and small seeds showed the highest germination rate compared to the flat form.

For the interval of germination, there is not a significant difference between the three forms of seeds of the two varieties *Ortanique* and *Murcott Honney*, their seeds gave all an average interval of three days. Unlike the other two genotypes, there is a statistically significant difference. The variety *Nadorcott* presented two groups ***a*** which includes the normal seeds and ***ab*** the small and flat seeds, while for the variant of *Murcott* M104 three statistical groups were distinguished ***a*** which includes the normal seeds, group ***ab*** for the small seeds and ***b*** for the flat seeds (Fig. 5).

Stem and root growth rate: For the growth speed of the stem during the first two weeks, the small seeds of the variety *Ortanique* are characterized by a better aerial growth, followed by the normal and flat seeds. For the *Nadorcott* variety, there is no significant difference between the three seed forms, they all gave the same growth rate. For the variant of *Murcott* M104, the small seeds were characterized by a better stem growth. Also, the small seeds gave a fast growth rate compared to the flat and normal seeds. At the fourth week, the stem growth rate decreased for all four tangerine varieties, this can be explained by the depletion of growing medium for the tangerine varieties during the first two weeks.

The seed forms that are characterized by better root growth rate in the first two weeks are small seeds for *Ortanique* variety, normal seeds for varieties *Nadorcott* and *Murcott honney*. The flat seeds of the variant of *Murcott* M104 showed a better growth rate, at the third week, the root growth rate decreased for the three seed forms of the four varieties studied and this can be explained by the depletion of the culture medium (Fig. 6 and 7).

Effect of size seeds on different parameters studied

According to a statistical comparison of the values of the studied parameters **(**Table 4**),** for the germination rate of the embryos there is a significant difference for the three forms of seeds, which detects two large groups, the group ***a*** including the flat form giving a maximum germination rate (87.69%) and group ***b*** including the two small and normal forms with an average germination rate of 84.68%. While there is no significant difference for the other variables studied (germination interval, stem growth rate (SGR1 and SGR2), root growth rate (RGR1 and RGR2) in relation to the tested seed form (small, flat and normal). The average values vary respectively as follows: 2.88 mm/d; 1.9 mm/d; 1.7 mm/d; 3.5 mm/d and 2.3 mm/d.

The results obtained showed a statistical variation of the variables according to the genotype and all depending on the seed shape (Table 5), the interaction between the seed shape and the variety studied varied for each character, the interaction is highly significant for the germination percentage ($P < 0.001$), it is significant for germination interval and root growth rate between the fourth and third week (RGR2), however the interaction is statistically insignificant for aerial growth rate during the four weeks (SGR1 and SGR2) as well as root growth rate between the second and first week (RGR1) with ($P > 0.05$).

Table 3: Growth rate of stem and root of four mandarin's varieties

Mandarin varieties	Growth rate (mm/day)			
	Stem		Root	
	SGR1	SGR2	RGR1	RGR2
Ortanique	3.70a	1.80b	3.80ab	2.30ab
Nadorcott	1.20b	0.60c	3.40b	1.60b
Variant of Murcott M104	2.20ab	1.40bc	1.90c	3.30a
Murcott honney	1.00b	0.60c	3.00bc	2.10b
Mean	2.03±1.23	1.10±0.60	3.03±0.82	2.33±0.71

Duncan's test with significance (5%)

SGR1: stem growth rate of seedlings in the second week, **SGR2:** stem growth rate of seedlings in the third week, **RGR1:** root growth rate of seedlings in 2nd week, **RGR2:** root growth rate of seedlings in 3rd week

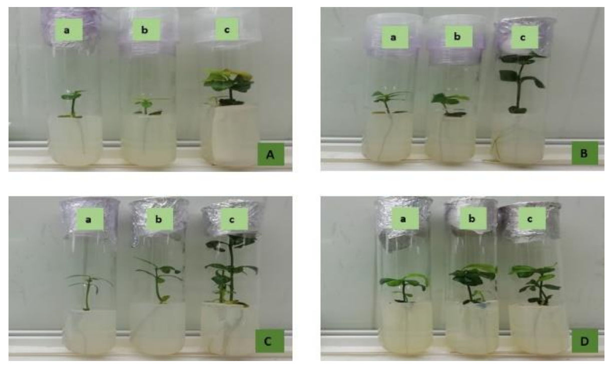

Fig. 3: Seedlings from immature embryo rescue of three types of seeds; a: normal; b: small; c: flat; for four varieties of mandarin's (A: *Nadocott*; B: Variant of *Murcott* M104; C: *Ortanique*; D: *Murcott Honney*).

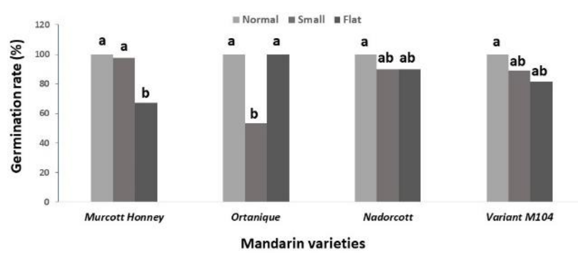

Fig. 4: Comparison of immature embryos germination rate according to the seeds size (normal, small and flat) of the mandarin's varieties.

Leaf analysis by flow cytometry

A substantial number of triploid plants have been recovered from immature embryos of citrus varieties in M1 medium containing GA3, through leaf analysis by flow cytometry technique. The triploidy rate varied according to the size of the seed and the variety studied.

Study of the genotype effect on triploidy: The variety *Ortanique* showed a higher triploidy rate than the other varieties with a percentage of 13.51%, followed by the variant of *Murcott* M104 and *Nadorcott* with an average percentage of 8.11 and 5.40% respectively. Then the variety *Murcott honney* which is in last place producing a low percentage of triploid vitroplants at about 2.70%. According to these results, mandarin varieties are able to give a very high triploidy rate but depending on the genotype studied (Table 6).

Study of the influence of seed size on triploidy: In the case for the variety *Ortanique*, 8.11% of the triploid plants were derived from small seeds compared to diploid hybrids, in contrast to the flat seeds which gave a percentage of 5.41% of triploid plants. The variant M104 also showed similar results due to the fact that only small seeds gave a high percentage of triploid plants in order of 8,11% and no triploid hybrids were regenerated from flat seeds. For the variety *Nadorcott*, there is an equality of percentage between the small and flat seeds, both had the same chance to give triploids. Lastly and the case of the variety *Murcott Honney* that was found in this study only one triploid hybrid, this last one was regenerated from the germination of a flat seed while the small seeds of this variety studied gave a percentage in the order of 2.70% and 0% for the small seeds.

Results showed the significant effect of genotype and seed shape on the percentage of triploidy of all the mandarins varieties studied, the variety *Ortanique* showed a very good ability to regenerate triploid hybrids in comparison with the other varieties and for the small seeds are the best to produce these hybrids sought in the case of the three varieties *Ortanique*, variant of *Murcott* M104 and the *Nadorcott* compared to the flat seeds giving low percentages of triploidy. Only the variety *Murcott honney* from which a single triploid hybrid was obtained from a flat seed (Table 7). The variety *Ortanique* appeared to be the best genotype to produce triploid *in vitro* plants, which are mainly derived from small seed germination.

The ploidy level profiles of the varieties: *Nadorcott*, *Murcott honney*, *Ortanique* and variant of *Murcott* M104, resulting from the immature embryo rescue are presented as histograms (Fig. 8). The comparison of the plants tested, which are derived from *in vitro* immature embryo rescue, with a triploid control profile (Moroccan Mandarin Hana) and a diploid control profile (Fig. 9), allows us to conclude the amount of DNA in the *in vitro* plantlet tested and thus its ploidy level.

The Seedlings confirmed as triploid by flow cytometry are transplanted into pots in a greenhouse. After two to three weeks, they are transplanted into black plastic bags. Thus,

Table 4: Multiple comparisons of the average values of the different parameters studied according to the shape of the seeds (small, flat and normal)

Variables	Seed shape			
	Small	Flat	Normal	Mean
Germination rate (%)	78.67b	87.69a	87.68a	84.68±5.20
Germination interval (days)	3.00a	2.79a	2.87a	2.88±0.11
Stem Growth Rate (mm/days)				
SGR1	2.51a	1.72a	1.60a	1.93±0.49
SGR2	1.85a	1.65a	1.94a	1.76±0.15
Root Growth Rate (mm/days)				
RGR1	3.62a	3.14a	3.89a	3.50±0.38
RGR2	2.64a	2.37a	2.23a	2.36±0.21

SGR1: stem growth rate of seedlings in the second week, **SGR2**: stem growth rate of seedlings in the third week, **RGR1**: root growth rate of seedlings in 2nd week, **RGR2**: root growth rate of seedlings in 3rd week.

Table 5: Multiple comparisons of genotype*seeds size interaction

Variables	CV (%)	F Value	Pr > F
Germination rate (%)	20.48	74.22	<0.0001***
Germination interval (days)	21.08	2.33	0.0226*
Stem Growth Rate (mm/days)			
SGR1	178.37	0.40	0.9190
SGR2	89.17	1.07	0.3870
Root Growth Rate (mm/days)			
RGR1	60.61	1.45	0.1817
RGR2	76.81	2.25	0.0281*

SGR1: stem growth rate of seedlings in the second week, **SGR2:** stem growth rate of seedlings in the third week, **RGR1:** root growth rate of seedlings in 2nd week, **RGR2:** root growth rate of seedlings in 3rd week.

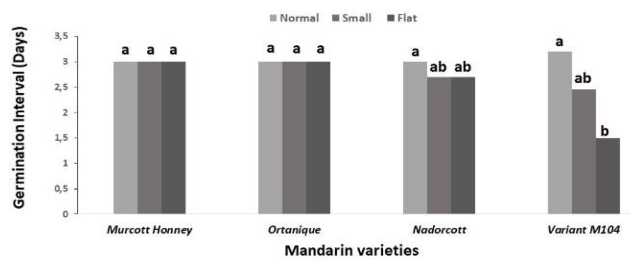

Fig. 5: Comparison of immature embryos germination interval according to the seeds size (normal, small and flat) of the mandarin's varieties.

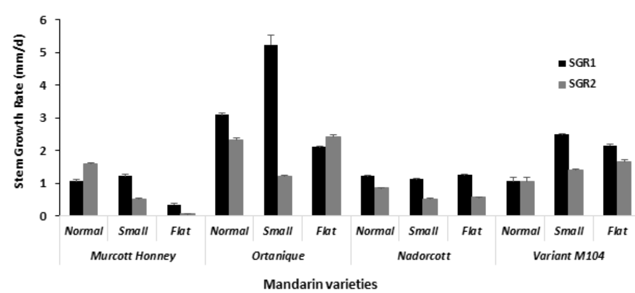

Fig. 6: Comparison of the Stem growth rate for the four mandarin varieties. SGR1: Stem growth rate during two weeks; SGR2: Stem growth rate during three weeks

the triploid seedlings will be grafted and transplanted into large pots (Fig. 10). They were placed in a greenhouse under conditions of temperature 25±2°C and humidity 80%.

Discussion

The average germination percentage was statistically different among the four mandarin varieties, suggesting an important distinction in the mechanisms controlling germination in these citrus cultivars. This finding is similar to those found by (Fucik *et al*. 1974,1978); Orbović *et al.* (2013) who found statistical differences for the germination rate of the cultivated seeds in citrus. Similarly, the statistical distinction of germination rates among the studied genotypes as well as stem growth; they may be related to the existence of seed coat during the cultivation. This was proved by Cimen *et al.* (2020) and Azim *et al*. (2013) who indicated that the shoot formation of *Citrus sinensis* is immensely related to the presence of seed coat during *in vitro* culture, which leads to a reduction in the germination percentage. In this study and according to mandarin's varieties studied, the germination rate varied from 55 to 98% for the small seeds and from 66 to 100% for flat-shaped seeds, these results showed similarity some previous studies (Aleza *et al.* 2010a; Ennaciri *et al.* 2020; Hmimidi *et al.* 2020; Mahmoudi *et al.* 2020), but it was higher than found by Ollitrault *et al.* (1996b) who reported the same for small clementine seeds.

In citrus, many studies have demonstrated the significant importance of selecting the optimal *in vitro* culture medium to ensure successful embryo rescue (Hmimidi *et al.* 2020; Ennaciri *et al.* 2020; Mahmoudi *et al*. 2020). In studying the germination of immature and mature seeds, we found a significantly difference in their development as a function of time, between seed types (small, flat and normal). This is reported by several studies (Silvertown 1981; Gross 1984; Stanton 1984) indicating that the shape of the seeds has an effect on the interval and the rate of germination and that the development differed over time according to the seeds size (Singh *et al.* 2003; Mahmoudi *et al.* 2020). Indeed, embryo rescue depends on the stages of embryonic development as well as the composition of the nutritional environment (Esen *et al.* 1973). Similarly, the choice of the best male (Handaji *et al.* 2005) and female (Essalhi *et al.* 2020) parents has been the subject of several studies to optimize the triploidy rate. Triploid embryos are found in small seeds that do not germinate conventionally under greenhouse conditions (Cuenca *et al.* 2010). In this study, triploid embryos were contained in seeds between 1/4 and 1/5 smaller than normal seeds (Table 1). The Embryo rescue from these small seeds is necessary to achieve higher germination rates (Navarro *et al*. 2002; Cuenca *et al.* 2010) indicating that triploids were obtained from underdeveloped seeds and possessing a size between 1/3 and 1/6 smaller than normal seeds (Esen *et al.* 1973; Thorpe *et al.* 1994; Aleza *et al.* 2010a). Likewise, spontaneous triploids have been detected in the progeny of Eureka lemon and lime, ruby sweet orange, and imperial grapefruit (Husband *et al.* 2004). The results of the latter studies confirmed that seed size is highly correlated with ploidy level. Indeed, the size of the seeds, carrying triploid embryos, of the order of l/3 to l/6 is smaller than those carrying diploids (Esen *et al.* 1973; Handaji *et al.* 2005).

Table 6: Percentage of diploid and triploid seedlings from *in vitro* germination of abnormal seeds (flat and small) analyzed by flow cytometry

Mandarins varieties	Percentage of plantlets	
	Diploids	Triploids
Ortanique	86.49	13.51
Variant of Murcott M104	91.89	8.11
Nadorcott	94.59	5.40
Murcott Honney	97.30	2.70
Total Mean	92.57±4.61	7.43±4.62

Table 7: Percentage of polyploid plants analyzed by flow cytometry of the four varieties studied according to seed shape.

Mandarins varieties	Percentage of polyploids		
	Seeds shape	Diploids	Triploids
Ortanique	Small	40.54	8.11
	Flat	45.95	5.41
Variant of Murcott M104	Small	48.65	8.11
	Flat	45.95	0.00
Nadorcott	Small	56.76	2.70
	Flat	35.14	2.70
Murcott Honney	Small	54.05	0.00
	Flat	43.24	2.70
Total Mean		46.28±6.99	3.72±3.21

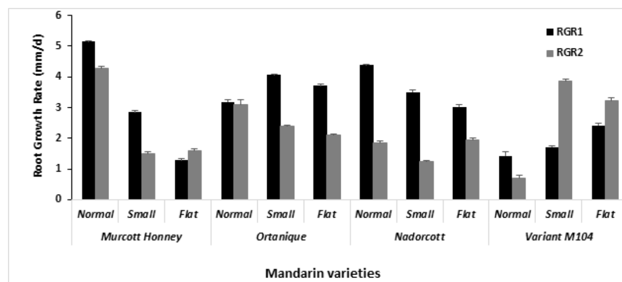

Fig. 7: Comparison of the root growth rate for the four mandarin varieties. **RGR1:** Root growth rate during the first two weeks; **RGR2:** Root growth rate during three weeks.

The probability of obtaining triploid citrus hybrids spontaneously is higher than 5% (Handaji *et al.* 2005). The frequency of unreduced megagametophyte production is a function of maternal genotype, and this information should be known before beginning extensive breeding programs (Aleza *et al.* 2010a). Furthermore, seed size is highly related to ploidy (Dalel *et al.* 2020). The study revealed that triploid hybrids were all obtained from partially developed seeds, mainly small seeds and this last character allows early selection of triploid plants.

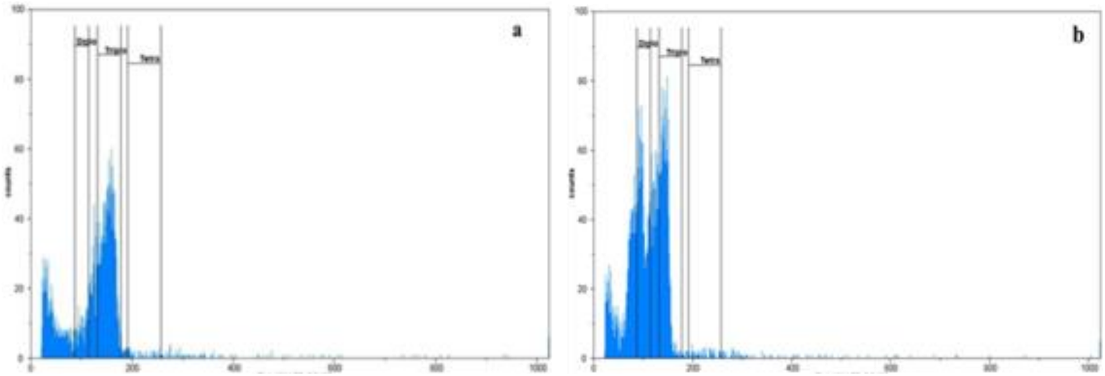

Fig. 8: Histograms detected by the flow cytometry technique for counting the amount of DNA; a: profile of a triploid *in vitro* plantlet; b: profile of a diploid *in vitro* plantlet

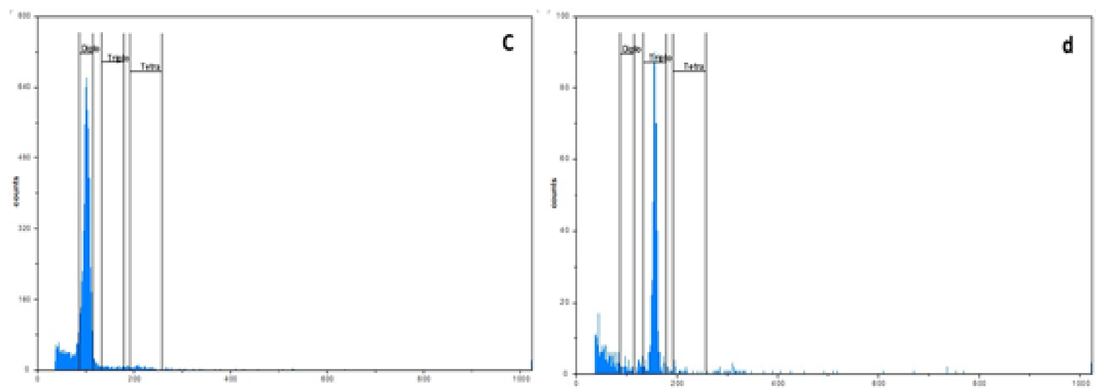

Fig. 9: Histograms generated by flow cytometry for counting DNA amounts; c: diploid control profile; d: triploid control profile

Fig. 10: Regeneration of triploid green seedlings. A: triploid seedlings from small seeds; B: seedlings transplanted in pots in the greenhouse; C: triploid seedlings from grafting of triploid mother plants; D: acclimatization of grafted plants in the greenhouse in large pots

Conclusion

The statistical variation of triploidy rate depends on two factors: genotypes and seeds shape. The embryo rescue linked to flow cytometry allowed to obtain and better detect triploid hybrids. The study of the genotype effect showed that only the variety *Ortanique* represented a good ability to produce triploids with a very high triploidy rate compared to the other genotypes and contrary to the *Murcott honney* which presented the lowest triploidy percentage. The seed shape effect revealed that triploid vitroplants were mainly derived from abnormal seeds (flat and small) especially the small seeds which have a high ability to produce and regenerate the desired triploid hybrids.

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Author Contributions

All authors have read and agreed to the published version of this manuscript.

Conflicts of Interest

The Authors declare that there is no conflict of interests that could possibly arise.

Data Availability

Data is available with the corresponding author

Ethics Approval

No applicable to this study

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