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Plant Cell Biotechnology and Molecular Biology 20(15&16):643–653; 2019 ISSN: 0972-2025

ANALYSIS OF PLOIDY LEVEL SEEDLINGS DERIVED FROM *in vivo* GERMINATION OF IMMATURE EMBRYOS OF ORANGES

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Received: 20 July 2019
Accepted: 25 September 2019
Published: 09 October 2019

Original Research Article

ABSTRACT

The triploid is a promising process in the creation of new seedless strains. *Pineapple* oranges and *Parson Brown* are characterized by the high quality that provides lots of seeds. However, our interest is a potential of two types as a female parent and as a seedless fruits hybrid production. Our study focuses on anomalous *in vivo* germination of these two varieties in order to obtain seedless triploid fruit varieties while analyzing their level of ploidy by flow cytometry. In fact, seeds are extracted from ripe fruits and are classified according to their morphological flat seeds, the small and the normal ones. Then, they were sown in greenhouse and in pans containing 50 % of peat and sand. The statistical analyses have shown that there is a significant difference between the form of seeds and the two varieties for all the outcomes studied. The germination rate has ranged from "20%" to "90%" depending on the normal shape and the abnormal one. Likewise, after seven weeks, stem growth has fluctuated from "4,90" to "7,40" with a comparatively low value in terms of smaller grain of the two varieties. With regard to the average germination time, it was significant for the shape of the seed and non-significant between the two varieties whose time is the highest to the small shape. Likewise, flow cytometric analysis of somatic embryogenesis allows to their level of ploidy to be determined. In fact, the rate of triploidy varies from "0%" to "9,5%" for *Parson Brown* and *Pineapple* varieties respectively. While the rate of haploidy was "2,5% for the flat shape of *Parson Brown*. This way of *in vivo* germination of immature oranges embryos may complement the in – *vitro* technique of rescue by mastering well the favorable conditions of germination.

Keywords: Orange; ploidy; haploidy; triploidy; flow cytometry; *in vivo* germination; immature embryo rescue.

INTRODUCTION

Citrus is one of the most predominant fruit crops grown globally, with great nutritional, medicinal and economic importance. Sweet orange, [*Citrus sinensis (L.) Osbeck*] are essentially diploids (2n = 2x = 18) and has been increasingly appreciated by consumers worldwide owing to its juice, abundant anthocyanin and other health-promoting compounds. The Citrus world's production is predominated by oranges with 51,8 million tons in 2019 [1], in fact, orange trees may be marketed in

the form of fresh fruits or turned into juice [2]. In *Citrus sinensis*, different factors are associated with parthenocarpy such as polyploidy, male sterility, self-incompatibility, abortion of ovule or embryo sac [3-9], all of which could promote seedless fruit production. As a matter of fact, *Pineapple* is an economically important plant, its production is between 16 – 19 million tons [10-12]. *Pineapple* and its juice is non-alcoholic drink and the demand continues to rise mainly due to increasing awareness of its health benefits [13]. *Pineapple* also contains polyphenolic compounds and possesses antioxidant activity [14]. Its pulp is juicy and fleshy with the stem serving as a supporting fibrous core, an excellent source of antioxidant [15]. Furthemore, *Parson Brown*' sweet orange is a well-known early-maturing seedy variety whose importance has slowly declined over time. Its usefulness as a commercial variety has been based on its better juice color and soluble solids [16]. Thus, these two types are classified among the most exploited varieties in the industries of juice in Florida [17,18]. [19] indeed authors shows that the presence of seeds in the orange causes a bitter taste in the juice. Hence the interest of looking for seedless orange varieties. Several methods has been developed to obtain citrus triploidy [20-23]. Triploid hybrids has been recovered from immature embryos of *Pineapple* and *Parson Brown* orange varieties based on of *in vitro* embryos rescue followed by the analysis of ploidy level by flow- cytometry [24]. It is seen that this conventional technique require material means and chemical products that's why the interest of this study is directed towards a looking for more simple cheaper technique. Germination *in vivo* of immature embryos in order to obtain triploid hybrids with seedless fruits.to the effect that the majority of embryos cannot survive *in vivo* or become dormant for long periods of time [25]. In the past, the determination of ploidy level in plants was limited to karyotypical assessments counting the number of metaphase chromosomes [26]. However, the somatic metaphase chromosomes of all citrus species are rather small (2–4 μm) [27]. In addition, morphologically similar chromosomes and the paucity of effective chromosome- specific markers make it difficult to accurately discriminate individual chromosome pairs during karyotype analyses that depend solely on conventional cytogenetic approaches in citrus. As an alternative to chromosome counting, other techniques not requiring actively dividing cells have been reported by several authors, such as the estimation of leaf stomatal density and size in *Citrus clementine* [28]. In general, leaves of *in vitro* grown plants exhibited higher stomatal frequencies than those of *in vivo* plants. The correlation between high stomatal density and extent of water loss had been well studied in tissue culture derived plants. The major reason for high mortality rate during *ex vitro* transfer relies on excessive water loss due to high stomatal densities [29]. A method to calculate the mitotic index and ploidy level of citrus callus, based on the analysis of cell size that can be used as a morphological marker to calculate these parameters for citrus callus was developed. Finally, flow cytometry has arisen as a far more reliable methodology for the determination of ploidy level. This article seeks to study the *in vivo* germination of the normal and anomalous seed shape of two varieties of *Parson Brown* orange tree and *Pineapple* so as to compare their germination, growth, triploidy and equally analyze its ploidy level by flow- cytometry in order to identify triploidy hybrids with seedless fruits.

MATERIALS AND METHODS

Plant Material

Two types of orange (Pineapple and Parson Brown) were used for *in vivo* germination of their mature and immature embryos. Samples were harvested at the level of the collection of National Institute of Agronomic Research (INRA), during the period November to February (Fig. 1).

Method and Culture Medium

At the maturity stage, all seeds were extracted from ripened fruit and were classified into two categories according to its normal morphology: (seeds fully grown) and abnormal one (seeds partly grown of flat or small shape) (Fig. 2). Under the greenhouse, the abnormal seeds (flat, small and normal) were positioned in basins containing 50% of peat and 50% of sand.

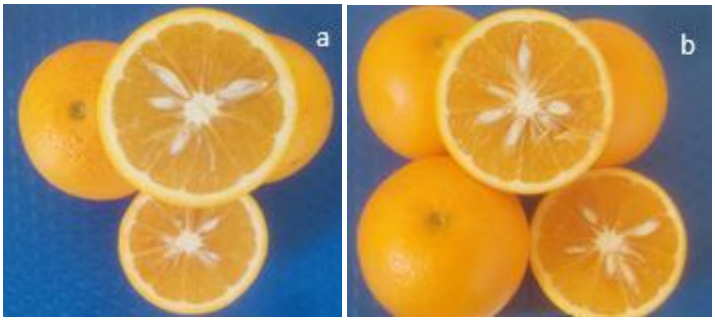


Fig. 1. The two types of orange. (a: *Pineapple* and b: *Parson Brown*)

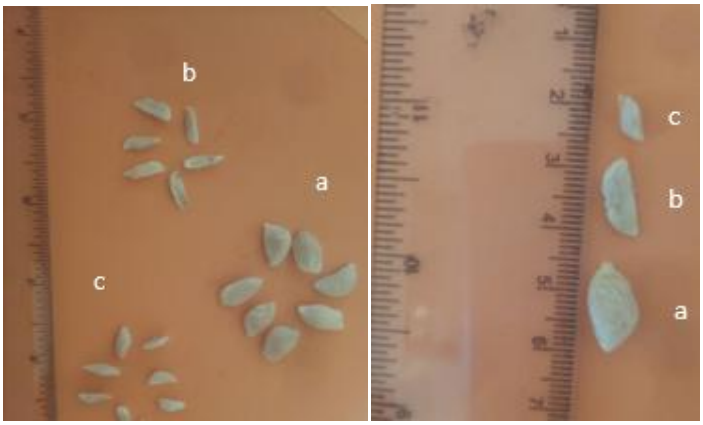


Fig. 2. Seeds extracted from oranges and classified according to their size (Witness, flat, and small)

Outcomes Studied

The outcomes studied are:

The rate of Germination (%) was calculated by the following relation:

$$" \frac{NGSX100}{NTS} "$$

NGS: Number of germinated seeds
NTS: Number of total seeds.

The interval average of germination: was calculated following relation:

IAG = Dpc – DG

Dpc=Date of placing in culture
DG= Date of germination.

The average number of plantlet's leaves: was calculated during each week.

The length of the rod: was calculated each week.

Acclimations of Plantlets

Plantlets were transplanted in jars containing 50% of sand and 50% of peat in the greenhouse, after; they were premastered in bags in order to foster their growth.

Flow Cytometry Analysis

Ploidy levels of seedlings from the rescue of immature embryos were evaluated by flow cytometry using a Partec II cytometer. The leaf tissues of each sample were finely cut in the presence of the triploid control (Moroccan mandarin Hana) with a razor blade, in a Petri dish containing 0.5 mL of phosphate-buffered saline (PBS) buffer, dithiothreitol 1 mg/L and 0.1% Triton × 100. The core suspension was filtered using a 40 µm nylon filter. Half a milliliter of a propidium iodide solution at 1 mg/Ml was added

and the mixture was incubated at room temperature for 5 min. This fluorochrome specifically binds to the DNA and under ultraviolet (UV) excitation at 365 nm; the fluorescence intensity re-emitted by the nuclei is proportional to the amount of visible DNA on the abscissa axis. Measurements of several thousand nuclei were retranscribed as a histogram.

Statistical Analysis of Data

The obtained data has been analyzed with the help of XLSTAT software program. The analysis of variance and the classification of medium- sized have been carried according to Tukey's test at 5% threshold. Thus, the Dunett's test has been allowed to statistically validate the variance of the studied parameters, the average time, the average number of leaves and the average length of rod for the two types *Parson Brown* and *Pineapple*.

RESULTS AND DISCUSSION

Study of the Effect of the Seed Shape and Genotypes on the Variables of Germination

Rate of germination (%): The rate of *in vivo* germination varied from "20%" to "90%" respectively to abnormal and normal seeds, (Fig. 3) have highlighted the influence of the seed shape (normal small and flat) and the effect types of genotype on the rate of germination. In fact, the germination percentage of normal (witness) ranged from "80%" to "90%" respectively to *Pineapple* and *Parson Brown* followed by those of the small shape 49% and 28% and finally, the flat shape with 30% and 28%.

The average time of germination (days): The statistical analysis was showed that there isn't a significant difference for the normal and abnormal shape. Furthermore, the higher time was for the small and the shortest was that the witness of the two types. In fact, the average time of germination (day) of control has recorded 33 days for the two genotypes *Pineapple* and *Parson Brown* followed by that of the flat form 70 days and the small shape 85 days of *Pineapple*, after, the small shape 89 days and the flat shape 93 days of the *Parson Brown* (Fig. 4).

Variation of average number of leaves in terms of the shape (small, flat and witness) of two verities of orange within the same period of observation: The average number of leaves have fluctuated from "4,90" to "7,40" for the whole shape of seeds. In fact, the average results values of the average number of leaves and the normal and abnormal shape of the two types of orange were shown to have a significant variation through

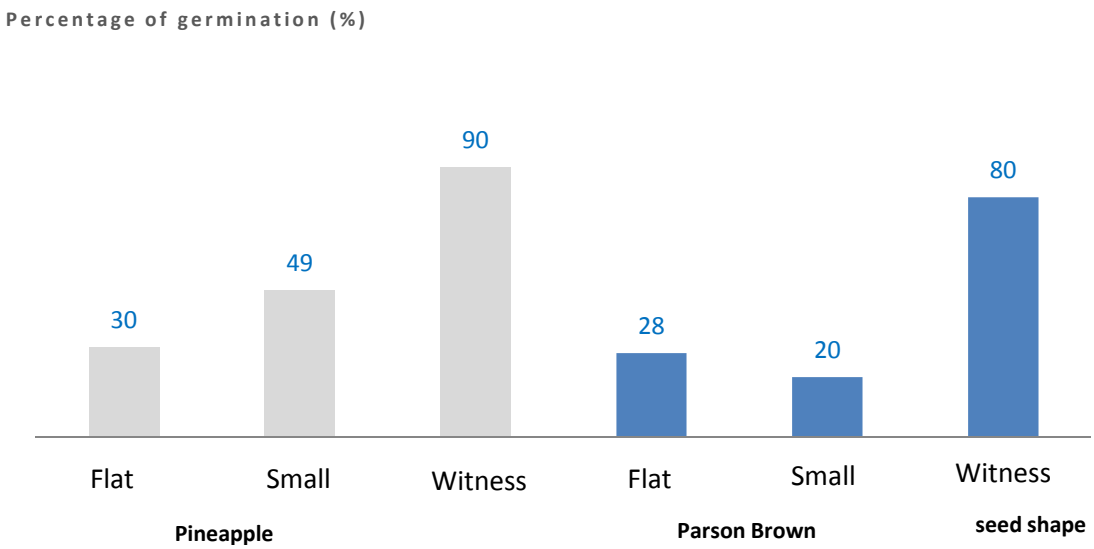


Fig. 3. Germination rate *in vivo* of normal and abnormal seed of *Pineapple* and *Parson Brown* orange

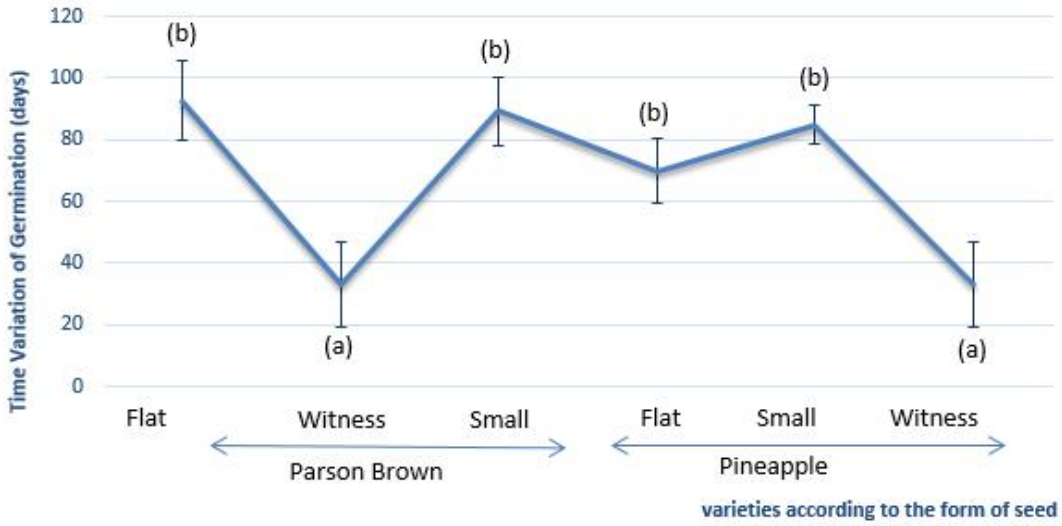


Fig. 4. Time variation of germination of two the verities (orange *Pineapple* and *Parson Brown*) based on the seed shape (flat, small and witness)

the weeks. The values which are relatively low was observed for the small shape of the two types. Furthemore, the (Table 1) was shown to have no significant variation for all the shapes of the seeds.

Table 1. Variation of average number of leaves for the normal and abnormal of of *Pineapple* orange and *Parson Brown*

	L 3 W
P. Brown /Witness	4,8000 a
Pineapple/Flat	4,4444 a
P. Brown /Flat	4,1667 a
Pineapple/Witness	3,6000 a
P. Brown/Small	3,0000 a
Pineapple/Small	3,1923 a
Pr > F(Model)	0,0906
Significative	No
Pr > F(Variety)	0,0906
Significative	No

L: Leave; W: Weak

The development of the rod for the seed shape (normal and abnormal) of oranges *Pineapple* and *Parson Brown*: After seven weeks, the development of the rod has ranged from "3,50" to "7,40" cm for the overall shapes, namely, the two different studied types including the normal and the abnormal. In fact, the results of the analysis variance have shown a significant effect of the variable (development of the rod) for the normal and abnormal shape. A maximum average of rod development has been observed at the flat shape of the *Parson Brown* type followed by the normal shape of *Pineapple*. However, the normal form of *Parson Brown* has detected a low growth and the small shape of *Pineapple* type has shown a weak development during the period of rise.

The average growth of the rod varies statistically according to normal and abnormal types of orange tree; the statistic treatment by Dunett's test was showed a significant difference during these seven weeks with regard to the 6^{th} and 7^{th}, there wasn't significant difference for the three forms of seed of the two types of orange tree *Pineapple* and *Parson Brown* (Table 2).

Analysis Result of Flow Cytometry

The analysis of flow cytometry of plantlets belonging to germination has allowed determining their ploidy level. The triploidy rate varied from "0%" to "9,5% "respectively for *Parson Brown* and *Pineapple*, while the rate of haploid was "2,5%" for the flat shape of *Parson Brown*. This way of germination *in vivo* of immature embryo of orange could complete the technique of embryos rescue *in vitro* by mastering well the favorable conditions of germination. In fact, "9.5%"of triploid hybrids and "90,5%"of triploid hybrids of the small shape has been recuperated from *Pineapple*, yet, the flat form has given us only

diploid concerning the *Parson Brown* type "2,5%" of haploid hybrids and "97,5%" of diploid hybrid, they have been obtained for the flat shape and "100%" of diploid hybrids for the small shape, which showed the genotype effect and the seeds' shape on the triploidy rate (%).

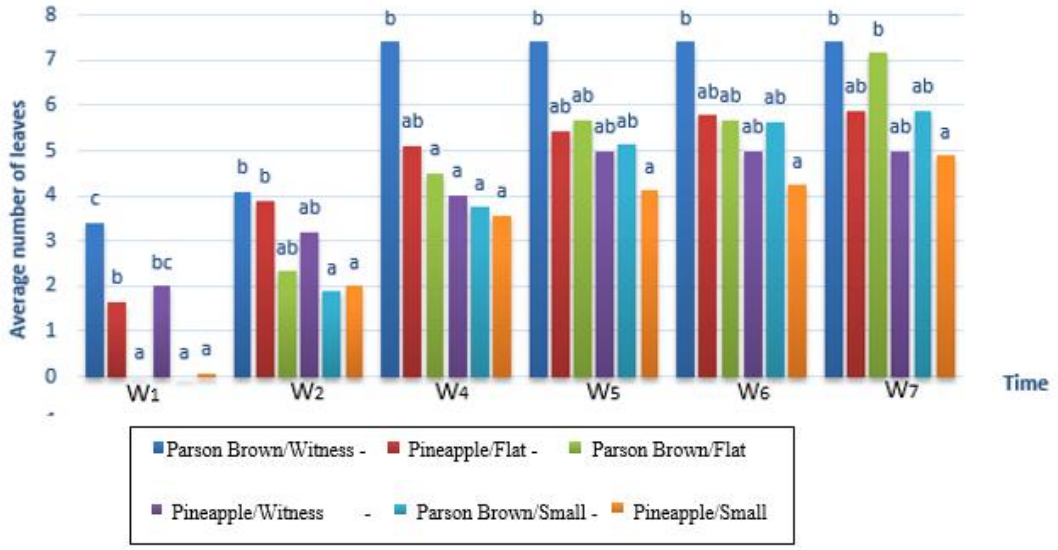


Fig. 5. Study of average number of leaves by seedling and by week during two months among the two types of studied orange *Pineapple* and *Parson Brown*

Two results read on the same graph are significative unless they are affected by the same letter

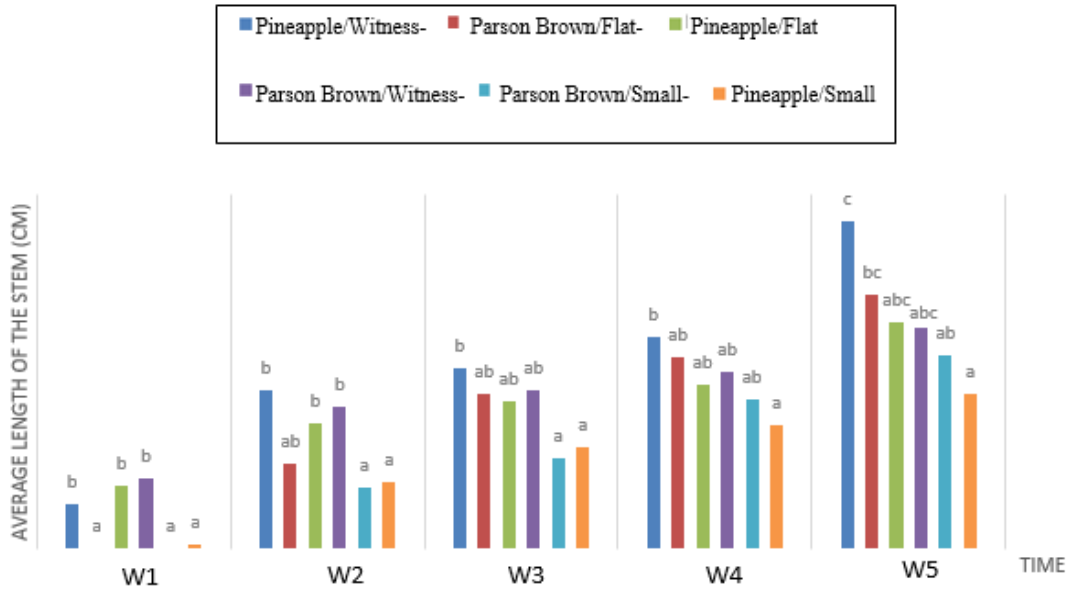


Fig. 6. Variation study of the average number length of the rod by the Tukey test for the two studied of type's *Pineapple* and *Parson Brown*

Two results read on the same graph are significative unless they are affected by the same letter

Table 2. Variation the average length of the rod for the normal and abnormal shape of oranges *Pineapple* and *Parson Brown*

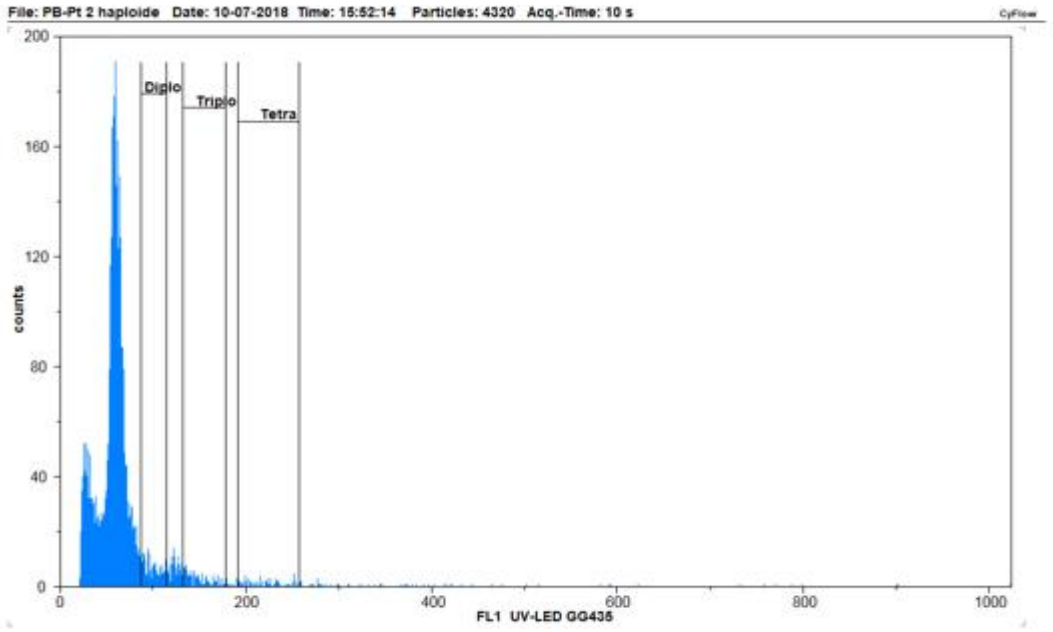
	LR6W	LR7W
Pineapple/Witness	7,4000 a	7,4000 a
P.Brown /Flat	6,4167 a	7,8333 a
Pineapple/Flat	6,0556 a	6,1111 a
P.Brown /Witness	5,0000 a	5,0000 a
P.Brown/Small	5,8125 a	6,8125 a
Pineapple/Small	5,1538 a	5,5192 a
Pr > F(Model)	0,4249	0,1226
Significative	No	No
Pr > F(Variety)	0,4249	0,1226
Significative	No	No

L: Length; R:Rod; W:Week

Table 3. Analysis of plantlets based on the germination *in vivo* of recuperated plants from immature embryos of oranges (*Parson Brown* and *Pineapple*)

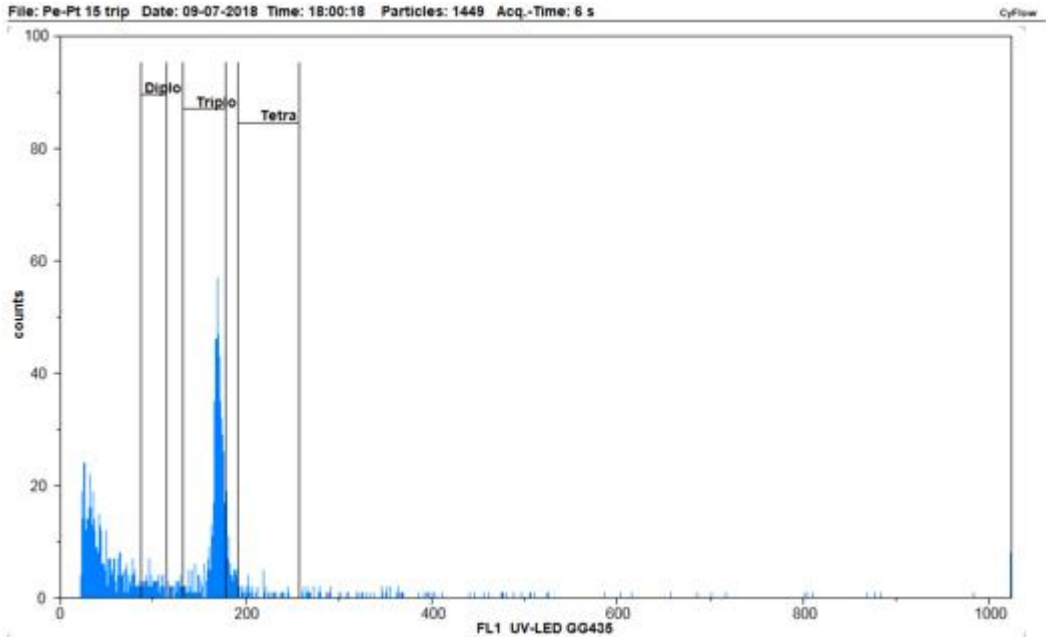
Cultivars	The seed shape	Percentage of ploidy		
		Haploid	Diploid	Triploid
P. Brown	Small	0,00	1,00	0,00
	Flat	2,5	97,50	00,00
Pineapple	Small	0,00	90,50	9,50
	Flat	0,00	100,00	00,00

The level ploidy profiles of the two types of *Parson Brown* and *Pineapple* of immature embryo rescue with that witness are displayed in the form of histogram (Fig. 7); the intensity of released fluorescence appearing on the axis of abscissa and the orderly number of cores.



Region	Gate	Ungated	Count	Count/ml	% Gated	Mean-x	CV-x %	Mean-y	CV-Y %
Diplo	<None>	213	213	-	4.93	99.81	8.58	-	-
Triplo	<None>	129	129	-	2.99	150.74	8.56	-	-
Tetra	<None>	73	73	-	1.69	221.43	8.63	-	-

A / case of analysis of a haploid hybrid



Region	Gate	Ungated	Count	Count/ml	% Gated	Mean-x	CV-x %	Mean-y	CV-Y %
Diplo	<None>	77	77	-	5.31	101.17	7.63	-	-
Triplo	<None>	586	586	-	40.44	168.17	5.18	-	-
Tetra	<None>	49	49	-	3.38	215.69	7.66	-	-

B / case of analysis of a triploid hybrid

Fig. 7. Histogram of flow cytometry for the counting of DNA quantity (A: haploid peak; B: triploid peak)

The results have detected a significant difference between the seeds shape and between the two types for all the studied variables. The rate of germination has been very low concerning the witness shape of the two genotypes. Further studies have shown that the witness seed grows very well. However, the small seeds are very difficult to be grown and need a method of embryos rescue by culture, very *in vitro* [30,31]. The treatment of black orris grain with geberrilic acid before the culture break the dormancy and improve its growth [32]. Other studies conducted on blonde oranges have shown that the growth of immature embryos *in vivo* is correlated especially with the age of the seed [33]. Like the case of tomatoes during a crop of immature embryos [34], the majority of embryos can't survive *in vivo* and become dormant during the long period. In general, it can't grow completely *in vivo* [35]. Thus, the immature embryos rescue constitude an important means for its growth so as to recuperate the triploidy hybrids because the right growth of immature embryos is fostered by the presence of acid hormone gibberellic and cytokinin [36]. Likewise, a study of germination *in vivo* of seeds on papaye type (*Carica – Papaye – L*) has revealed that the average time of germination depend on genotype as well as the treatment of embryos before the sowing by gibberellic acid may reduce the time of germination [37]. Moreover, the induction of caulogenesis has been recuperated from the normal and abnormal sowing *in vivo* for the two types including *Pineapple* and *Parson Brown*. But with a variable reactivity, which rely on genotype and even on the seed shape, after seven weeks, the growth of the rod rang from "3,5" to "7,4" cm for the overall form namely the same genotype for the three forms of grain (small, flat, and normal) which the growth of its rod *in vitro* has varied from "2" to "2,5" cm. Besides, our study has detected the effect of genotype and the form of grain on the rate of

triploidy *in vivo*. In fact, we have obtained a very low rate of triploidy and haploid, as opposed to results found in the same genotype *in vitro* [24], an important rate of triploidy. However, any hybrid haploid has been observed. Few researches carried out on the production of hybrids triploidy from the culture *in vivo* of immature embryos. Also, authors [38-40] have shown that in rare cases, hybrids triploidy may be found in sowing under green house, like the case for "*Winola*" mandarin orange [41], but thanks to the immature embryo rescue *in vitro*, the hybrids triploidy may be recuperated like the case of the two orange *Pineapple* and *Parson Brown* [24]. Moreover, [42-44] have stated the triploidy embryos are preferably found in the seeds between 1/3 and 1/6 smaller than the normal one. In addition, studies on Clementine tree demonstrate that the triploidy embryos are generally found between "52" and "62%" on the small seed than the normal ones [45]. On the other hand, the normal seed of pamelos has resulted 1.4% of hybrids haploid and "72%" hybrids triploidy "25%" diploid [46,47] also showed that the environmental conditions affect the frequence of recovering triploidy. In fact, the diploid female genotype has a strong influence on the number of seed by fruit in the rate of triploidy.

CONCLUSION

This study led us to find triploid and haploid hybrids via the technique of *in vivo* germination of abnormally shaped seeds (flat and small). In fact, "9.5%" of triploid hybrids were recovered from the Pineapple variety and "2.5%" of haploid hybrids were obtained for the flat shape of the *Parson Brown* variety, consequently, this process of *in vivo* seed germination of abnormal orange could complement the *in vitro* rescue technique while exploiting the favorable conditions of germination.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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