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RESEARCH ARTICLE

DEVELOPMENT OF A RELIABLE AND REPRODUCIBLE TEST FOR THE OVULE FERTILITY STUDY OF SOME MANDARIN GROUP VARIETY (*CITRUS RETICULATA*) THROUGH MANUAL AND OPEN POLLINATION

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ABSTRACT

In Mandarin group, the ovule fertility represents the ability of a variety to produce seed in the fruit. This criterion could be used for the distinction between new varieties from irradiation and which are characterized by a great similarity with the parent varieties. The aim of this work is the research of an estimation technique of the ovule fertility in some variety of Clementine and Mandarin via estimating the seed number produced in manual and open pollination conditions. Three experiments were studied: hand pollination with different concentration of pollen, estimating the stigma receptivity by the percentage of pollen germination in vivo and in vitro and estimating the seed number produced by fruit under open pollination conditions. The results obtained showed that the stigma receptivity decreases when we take away from the anthesis stage. The seed number decreases when pollination was carried out six days after anthesis. In terms of pollen concentration, our results revealed an increase of seed number with the increase of pollen concentration. However, the open pollination conditions, allowed an important distinction between the ovule fertility degrees of the varieties tested. Our results demonstrate the importance of the open pollination technique in the determination of ovule fertility in the Mandarin group of citrus fruits, and therefore, a clear distinction between the varieties of this group.

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INTRODUCTION

Citrus are one of the cultures the most important in economic terms in the world (Benkabilia and al., 2008). The global trade of Citrus fruit demands high cosmetic and organoleptic characteristics; in recent years, seedless fruit has been one of the main requirements of the market of citrus fruits and one of the most important characteristics as regards the quality of fruits for consumption in expenses (Gambetta and al., 2013). The presence of a large number of seeds in citrus fruits is a big hindrance in consumer acceptability. Seed lessness is a desirable characteristic of both fresh and processed citrus markets. Therefore, development of seedless fruit cultivars has become a major goal for fruit breeders around the world (Yamamoto, 1995; Ye and al., 2009). Among the breeding methods for developing seedless cultivars are the production of

triploid hybrid seedlings and creating mutants by irradiation characterized by low ovule fertility (Yamamoto, 1995). The varieties from irradiation are characterized by the ability to produce seedless fruit. Thus, this ability was located just in the varieties comes from irradiation. The only criterion allowing the distinction between the generated varieties with the mother varieties is the reduced of ovule fertility, while the rest of their characteristics stand generally compliant with the original varieties, the case of tango varieties from the Nadorcott Mandarin irradiation (Roose and Williams, 2008).

The estimate of the ovule fertility degree is necessary for the distinction between the parent varieties and varieties come from irradiation. Generally, the ovule fertility is estimated by the ability of the variety tested produce seeds. Thus, this ability is conditioned by parent used for pollination. Indeed, Philip

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and *al.*, 1961 studying the effect of the pollen source on the seed number of variety Robinson and Osceola as the other varieties, found that the seed number registered in these varieties varies depending on the pollen origin.

De Lange and al., 1974, showed that in the Ortanique Mandarin variety, pollen of Valencia Late induces less seed than the pollen of Minneola tangelo. In the presence of pollen of this variety, Ortanique Mandarin records a high seed number. *Nadori and al.*, 1996, have shown that the pépinisation phenomenon in citrus differs considerably depending on the variety pollinated for some pollinator and according to a pollinator for the same variety used as female. Indeed, Nadorcott Mandarin (Afourer) is very sensitive when it is pollinated by pollen of Nova, Ortanique and Fortune. However, Sidi Aissa Clementine, Valencia late and Salustiana induced little of seeds in this variety. At Nova variety, there are lots of seeds when it is pollinated by pollen of Ortanique, Afourer and Sidi Aissa Clementine. However, pollen of Fortune induced an average seed number at Nova Mandarin. When Nova Mandarin is pollinated by pollen of Salustiana varieties, the fruits are seedless (without seeds). Also, under hand pollination conditions, it is found that the seed number is influenced by the pollen number used (*Brown and krezdorn*, 1969) as well as the physiological stage of the flower during pollination (*Mesejo and al.*, 2007; *sanzol and herrero*, 2001). *Masashi and al.*, 1995, showed a highly significant correlation between the seed number product at the fruit from hand pollination and the fruit from open pollination. Consequently, the degree of ovule fertility can be estimated from the seed number of open pollination. It is apparent from the research cited as the seed number at tests out-crossing for some variety remains unstable and depends on several factors, including pollination conditions, the pollen concentration and the physiological stage of the flower. In this way, the objective of this study is the development of a reliable and reproducible test for the ovule fertility study of some Mandarin group variety by using hand pollination and open pollination, this for a better distinction of some variety and therefore to be considered in LUPOV descriptors for the description and distinction the Mandarin varieties resulting from irradiation.

MATERIALS AND METHODS

This study was conducted in Morocco, in two regions: Gharb region, characterized by a sub-humid climate near the coast and Haouz, an arid and continental region.

Varieties used

The male varieties: NOVA Mandarin and NADORCOTT Mandarin; the female varieties: NULES Clementine, MARISOL Clementine, NOVA Mandarin and Mandarin. The tests were performed on trees 10 to 15 years.

Hand pollination

Collect the pollen

The flowers of varieties used as male parents, will be collected randomly according to the four cardinal directions of a tree, at

a growth stage of 61–62 on the Citrus BBCH phenological scale (*Agusti and al.*, 1997). Anthers separate from style and are placed in Petri dishes. Petri dishes are then placed at 25 °C during 24-48 h in obscurity. Anthers are observed to note the start of dehiscence and the liberation of pollen.

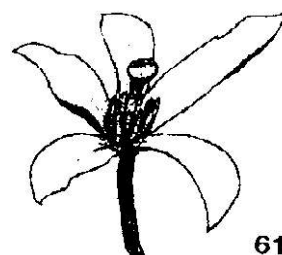


Figure 1 Growth stage of 61–62 on the Citrus BBCH phenological scale (*Agusti and al.*, 1997).

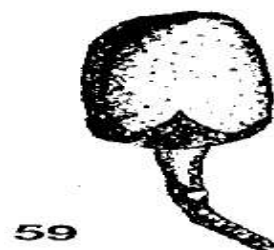


Figure 2 Stage 59 of the citrus BBCH scale Phenological (*Agusti and al.*, 1997).

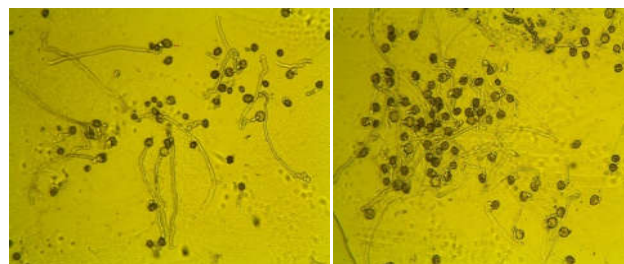


Figure 3 In vitro pollen germination of NADORCOTT Mandarin variety.

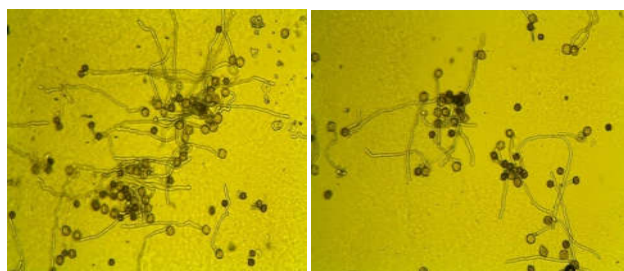


Figure 4 In vitro pollen germination of NOVA Mandarin variety.

Study of Pollen Viability

After dehiscence, pollen is then removed using a brush and sprayed into a microscope slide with 2 ml of Brew backer medium (*Brewbaker and Kwack*, 1963). Finally, the microscope slide should be placed at 24 °C with a 75 % RH during 20 hours. Percentage of pollen germination was estimated by calculating the average percentage of germinated pollen by microscopic observation.

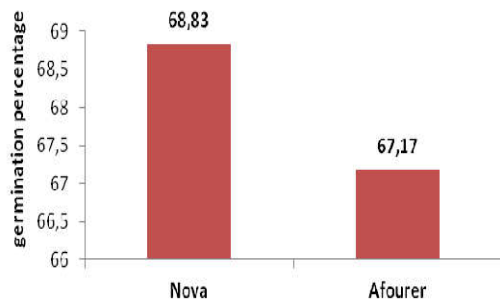


Figure 5 In vitro pollen germination percentages of NADORCOTT Mandarin and NOVA Mandarin

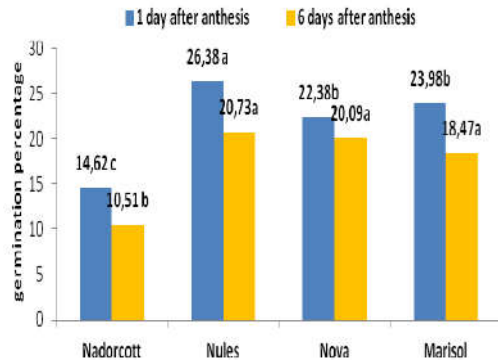


Figure 6 In vivo pollen germination percentages of NADORCOTT Mandarin in four varieties stigma, 1 day and 6 days after anthesis.

For the same dates, varieties followed by the same letter are not significantly different at the 5% level (Duncan Test).

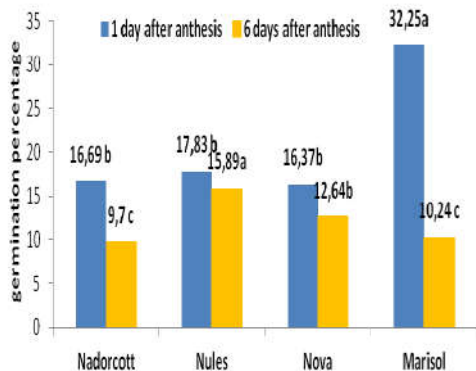


Figure 7 In vivo Pollen germination percentage of NOVA Mandarin in four varieties stigma, 1 day and 6 days after anthesis.

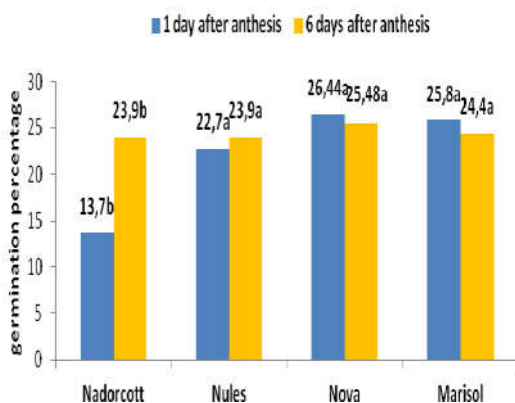


Figure 8 In vivo pollen germination Percentage of NADORCOTT Mandarin in four varieties stigma, 1 and 6 days after anthesis.

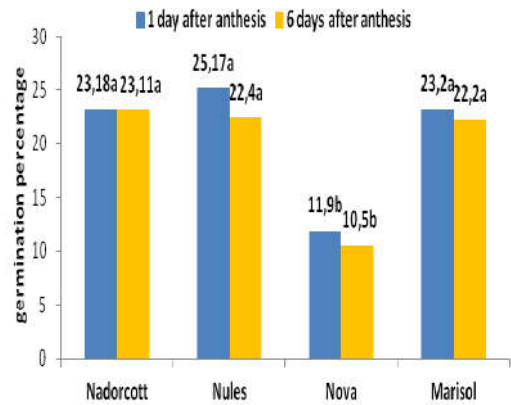


Figure 9 In vivo pollen germination percentage of NOVA Mandarin at four varieties stigma, 1 and 6 days after anthesis.

For the same dates, varieties followed by the same letter are not significantly different at the 5% level (Duncan Test).

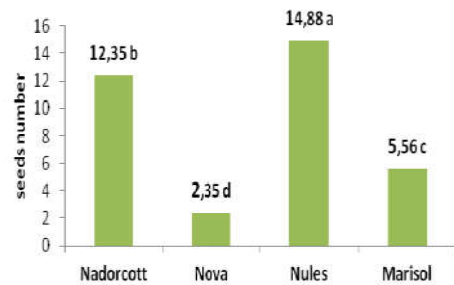


Figure 10 Seed number recorded in four citrus varieties in open pollination conditions.

Table 1 Seed number recorded in variety NULES Clementine at Gharb region depending on male pollinator, pollination date and pollen concentration.

Variety	Site	Male		Date		Concentration	
		Nadorcott	NOVA	1 day	6 days	saturation	100g
Nules	Gharb	29a	16b	25 a	20 b	24 a	21 b

Table 2 Seed number recorded in variety NULES Clementine at Haouz region depending on male pollinator, pollination date and pollen concentration.

Variety	Site	Male		Date		Concentration	
		Nadorcott	NOVA	1 day	6 days	saturation	100g
Nules	Haouz	23a	17 b	30a	9b	23a	17 b

Table 3 Seed number recorded in variety MARISOL Clementine at Gharb region depending on male pollinator, pollination date and pollen concentration

Variety	Site	Male		Date		Concentration	
		Nadorcott	NOVA	1 day	6 days	saturation	100g
Marisol	Gharb	9a	6b	8 a	6b	10a	4b

Table 4 Seed number recorded in variety MARISOL Clementine at Haouz region depending on male pollinator, pollination date and pollen concentration.

Variety	Site	Male		Date		Concentration	
		Nadorcott	NOVA	1 day	6 days	saturation	100g
Marisol	Haouz	13a	9 b	13 a	9 b	14 a	8 b

Emasculation flowers

One day before anthesis or stage of 61–62 on the Citrus BBCH phenological scale (Agusti and al., 1997), 100 flowers for every tree used as female parent, will be emasculated and

bagged to avoid self and free pollination (Moss and *al.*, 1972; Guardiola and *al.*, 1984).

Effect study of the pollen concentration and the pollination date on the seed number produced or ovule fertility degree

One day and 6 days after anthesis or stage 59 of the citrus BBCH scale phenological (Agusti and *al.*, 1997), the emasculated flowers are pollinated with pollen of male varieties, (100 pollen per flower and saturation concentration), by using a brush. To determine the concentration 100 of pollen, method suggested by alkamine and Giorlomi, 1959 was used. The pollen of varieties used were placed on the flowers were emasculated. The number of seeds is counted after harvesting a month before fruit maturation.

Effect study of stigma receptivity on the seed number produced or ovule fertility degree

For each pollination date, 10 flowers per variety will be removed 48 h after pollination and fixed with FPA (10% formaldehyde, 10% propionic acid, 80% ethanol at 70%). The percentage of pollen germination on the stigma will be calculated by counting at least 100 per pollinated flower. Pollen was considered to be germinated when the pollen tube length is longer than the pollen diameter. This is done by extracting the germinated pollen in the laboratory using the microscope (Mesejo and *al.*, 2007).

Open pollination

Three trees by male and female variety are used. No bagging or emasculation of flowers has been practiced. A month before maturity, 50 fruits per cardinal orientation was collected from each tree. The extraction of the seed is made in the laboratory and the number of seeds was counted. An average value per tree was calculated.

RESULTS

Pollen viability of male varieties

Pollen viability was estimated by the percentage of pollen germination in vitro. The figures show the pollen germination of two male varieties, NADORCOTT Mandarin and NOVA Mandarin. The variance analysis of pollen germination percentage in vitro of NADORCOTT Mandarin variety and NOVA Mandarin variety shows that there is no significant difference. From Figure 5, the pollen of two varieties used were recorded the germination percentage of 68.83% and 67.17% respectively for NOVA Mandarin and NADORCOTT Mandarin.

Effect of pollination date and pollen concentration on stigma receptivity in four varieties in the Gharb region

In vivo pollen germination percentage of variety Nadorcott Mandarin in four varieties stigma, pollinated of 1 and 6 days after anthesis

The variance analysis of pollen germination percentage of NADORCOTT Mandarin in four varieties stigma, pollinated of

1 and 6 days after anthesis, shows that there is a highly significant effect of female variety used and interaction female variety and pollen concentration. So, pollen germination of NADORCOTT Mandarin at stigma varies depending on the female variety used. Figure 6, shows that the pollen germination percentage of NADORCOTT Mandarin at female varieties stigma used, decreases after 6 days of anthesis.

Furthermore, the comparison of the averages percentages of pollen germination at the stigma, as well 1 day after anthesis as 6 days after anthesis, shows that pollen NADORCOTT Mandarin have a differential behavior in terms of germination percentage at the stigma of the female varieties used. Indeed, for 1 day after anthesis:

The highest pollen germination percentage of NADORCOTT Mandarin is recorded at stigma of NULES Clementine (26.38%). However, the lowest pollen germination percentage is recorded at the stigma of the variety NADORCOTT Mandarin (14.62%). 6 days after anthesis:

The lowest pollen germination percentage of NADORCOTT Mandarin is recorded at the stigma of the NADORCOTT mandarin variety (10.51%) against 20.73%; 20.9% and 18.47% respectively at the stigma of NULES Clementine, NOVA Mandarin and MARISOL Clementine.

In vivo pollen germination percentage of variety NOVA Mandarin in four varieties stigma, pollinated of 1 and 6 days after anthesis

The variance analysis of pollen germination percentage of Nova Mandarin in four varieties stigma, pollinated of 1 and 6 days after anthesis, shows that there is an effect highly significant of female variety used, pollen concentration and interaction female variety*concentration. So, pollen germination of Nova Mandarin at stigma varies depending on the female variety used, 1 and 6 days after anthesis.

Figure 7, shows that the pollen germination percentage of NOVA Mandarin at four female varieties used, decreases after 6 days of anthesis. Furthermore, the comparison of the average percentages of pollen germination at the stigma, as well 1 day after anthesis as 6 days after anthesis, shows that pollen NOVA Mandarin have a differential behavior in terms of germination percentage depending on the female varieties used. Indeed, for 1 day after anthesis:

The highest pollen germination percentage of NOVA Mandarin is recorded at the stigma of MARISOL Clementine (32.25%). However, the lowest pollen germination percentage is recorded at the stigma of variety NADORCOTT Mandarin, NULES Clementine and NOVA Mandarin. The values are respectively 16.69%, 17.83% and 16.37%. 6 days after anthesis: The lowest pollen germination percentage of NOVA Mandarin is recorded at the stigma of MARISOL Clementine (10.24%) and NADORCOTT Mandarin (9.7%). This percentage was 15.89% for NULES Clementine and 12.64% for NOVA Mandarin.

Effect of pollination date and pollen concentration on the stigma receptivity in four varieties in Haouz region

In vivo pollen germination percentage of variety Nadorcott Mandarin in four varieties stigma, pollinated of 1 and 6 days after anthesis

The variance analysis of pollen germination percentage of NADORCOTT Mandarin in four varieties stigma, pollinated 1 and 6 days after anthesis, shows that at 1 day of the anthesis, the very highly significant effect exists of female variety used and the concentration, against interaction variety * concentration proved non significant. For against, at 6 days of anthesis a very highly significant effect of the interaction variety * concentration is observed. This clearly shows that after 6 days of anthesis the varieties used, show a differential behavior vis-a-vis the concentrations used. Figure 8, show that the pollen germination percentage of NADORCOTT Mandarin at stigma female varieties used has been reduced after 6 days of anthesis. Furthermore, the comparison of average percentages of pollen germination at the stigma as well 1 days after anthesis as 6 days after anthesis, shows that the NADORCOTT Mandarin pollen have a differential behavior in terms of germination percentage at the stigma of female varieties used. Indeed, for one day after anthesis: The highest pollen germination percentage of NADORCOTT Mandarin is recorded at the stigma of NOVA Mandarin, MARISOL Clementine and NULES Clementine and we are respectively 26.44%, 25.8% and 22.7%. However, the lowest percentage of pollen germination is recorded at the stigma of the NADORCOTT Mandarin variety (13.7%). 6 days after anthesis: The lowest pollen germination percentage of NADORCOTT Mandarin is recorded at the stigma of the variety NADORCOTT Mandarin (12.36%) against 25.48%, 24.4% and 23.9% respectively at the stigma of MARISOL Clementine, NOVA Mandarin and NULES Clementine.

In vivo pollen germination percentage of variety NOVA Mandarin in four varieties stigma, pollinated of 1 and 6 days after anthesis

The variance analysis of pollen germination percentage of Nova Mandarin in four varieties stigma pollinated of 1 and 6 days after anthesis, shows that there is an effect highly significant of female variety used and pollen concentration. In addition an interaction variety * concentration proved significantly after 6 days of anthesis. Figure 9, show that for the four varieties used, the pollen germination percentage of NOVA Mandarin at stigma has been reduced after 6 days of anthesis. Furthermore, the comparison of the averages percentages of pollen germination at the stigma as well 1 days after anthesis as 6 days after anthesis, shows that NOVA Mandarin pollen have a differential behavior in terms of germination percentage depending on the variety used female. Indeed, for one day after anthesis: The highest pollen germination percentage is recorded at the stigma of NULES Clementine (25.17%), MARISOL Clementine (23.2%) and NADORCOTT Mandarin (23.18%). However, the lowest pollen germination percentage is recorded at the stigma of NOVA Mandarin (11.9%). 6 days after anthesis: the lowest pollen germination percentage is recorded at the stigma of NOVA Mandarin (10.5%) against 23.11%, 22.4% and 22.2%

respectively for NADORCOTT Mandarin, NULES Clementine and MARISOL Clementine.

Effect of hand pollination on the number of seeds produced at fruit

The variance analysis of seed number recorded at the two fruit varieties under conditions of hand pollination shows a highly significant effect of the male variety, pollination date, the pollen concentration and female variety used. Similarly, significant interactions were recorded four factors: Date* Site, Male * Date, Date * Variety female and female Variety * male variety. The seed number in the fruit is therefore conditioned by many factors: male variety, female variety and pollen concentration used. Tables 1 and 2 show that in two regions (Gharb and Haouz), the seed number recorded in NULES Clementine varies with male parent, pollination date and pollen concentration used. Indeed, the highest seed number is registered in presence of NADORCOTT Mandarin as a male parent, for pollination 2 days after anthesis and saturated pollen concentration.

Tables 3 and 4 also show that in two regions (Gharb and Haouz), the seed number recorded in Clementine Marisol varies with male parent, pollination date and pollen concentration used. Indeed, the highest number seed is registered in presence of Mandarin Nadorcott as a male parent, for pollination 2 days after anthesis and saturated pollen concentration.

Seeds number in the fruits under open pollination conditions

Variance analysis of seed number recorded at four varieties fruit in open pollination conditions shows a highly significant effect of variety and interaction variety * region, while, the region effect is mounted significant. Figure 10, shows that the seed number in fruits, in open condition pollination varies between the varieties used. Indeed, the averages comparison of seed numbers recorded in varieties allowed to identify four statistically different groups at the 5% level (Duncan Test). The first group is represented by NULES Clementine variety. This variety has recorded highest seed number, 14.88 seeds in fruit. The second group is represented by NADORCOTT Mandarin variety with 12.35 seeds in fruit. The third group is represented by MARISOL Clementine (5.56 seeds). The fourth group is represented by NOVA Mandarin (2.35 seeds).

Ovule fertility is estimated by the seed number produced under open pollination conditions. But under hand pollination conditions, many factors could be influenced on the seed number produced by varieties: pollinator parent, female variety, stigma receptivity, pollination date and pollen concentration used. The results obtained in this work suggest that receptivity of all female varieties used, which is estimated by in vivo pollen germination percentage, decreases when one move away the anthesis and pollen germination is differs from one variety to other. It is found that stigma receptivity depends on the anthesis stage and female variety. In agreement with work of Mesejo and al., (2007), which confirmed that Owari Satsuma has a short period of receptivity, two days after anthesis, unlike "Climenules" Mandarin and valencia which

have a long period of receptivity and a difference in the flower receptivity period between variety manifests. The differences in the flower receptivity period between species and cultivars were also reported in other culture, such as Pyrus, Malus and Prunus (Sanzol and Herrero, 2001).

Masashi and al., (1995) showed a positive correlation between seed number of hand pollination and open pollination. This indicates that the degree of ovule fertility can be estimated from the seed number of open pollination. Previously, Masashi and al., (1993) obtained a positive correlation between the average number of seeds per fruit in hybrids and their parents. Their results show that ovule fertility and sterility are hereditary characteristics.

Vithanage, (1991) studying the effect of pollen origin on seeds number of varieties "Ellendal" (tangor) Mandarin in cross-pollination mode, have use mandarin pollinators parents: "Imperial" Mandarin, "Siiverhili" Satsuma, "Emperor" Mandarin, "Dancy" Mandarin, "Murcott" Mandarin and "Valencia" orange; shows that the seed number of varieties "Ellendal" varies significantly with a parent used. Pollen of varieties "Murcott" and "Emperor" induced a high number of seeds against a reduced number of seeds is recorded in the presence the "Imperial" Mandarin pollen.

In addition, Ioannis and al., (2009) showed that pollination of Marisol Clementine by pollen of Nova mandarin produces fruit with 2.39 seed number. However, pollination of SRA 63 Mandarin by the pollen of Nova Mandarin produces fruit with 18.55 seed number. About the pollination date effect on the seed number in varieties, our study shows that the seed number reduced in pollination performed 6 days after anthesis, in agreement with the results of Mesejo *et al.* (2007) who noted the absence of seed production in Owari Satsuma when pollination performed 2 days after anthesis, while the "Clemenules" Mandarin and Valencia orange until 8 days after anthesis.

The seed number in Mandarin varieties also varies according to type of pollination. Our results show that under hand pollination conditions, reduction of seed number was observed. This reduction also varies according to male variety, female variety, the pollination date and the pollen concentration. In agreement with the results of Gambetta and al., (2013) found a relatively low percentage of seedless fruits in open pollination and 98% of seedless fruits in the hand pollination. About the pollen concentration used for hand pollination, the results obtained indicate an increase in seed number with an increase in pollen concentration used. These results are concurring with the work of Brown and Krezdorn, (1969). These authors showed that the seed number obtained for a 50 pollen concentration is significantly lower than that obtained for a 100 pollen concentration. So, the seed number varies depending on pollen concentration. The use of open pollination in this study allowed a significant difference in the ovule fertility degree of varieties used is based on the seed number produced by each variety. Our results at this point are concurring with those of Masashi and al., (1995) which showed the importance of open pollination in determining the ovule fertility in Citrus. Indeed,

this technique is not affected by pollen concentration or by the physiological state of flower.

CONCLUSION

In conclusion, the study of ovule fertility must take into account the pollen number, the stigma receptivity stage and pollination phase. The stigma receptivity depends on the female variety and pollination date. About the seeds number for the same variety, it varies depending on the male pollinator, pollen concentration and the pollination date. Under open pollination conditions, varieties studied are divided into four statistically different groups depending on the seed number per fruit produced by each variety. The results obtained allowed a clear distinction between varieties in terms of ovule fertility expressed by the seed number produced under open pollination conditions, while under hand pollination conditions the degree of fertility is influenced by pollen origin, region, pollen concentration and pollination date.

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