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UPOV

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TECHNICAL WORKING PARTY FOR FRUIT CROPS

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PARTIAL REVISION OF THE TEST GUIDELINES FOR MANDARIN
(DOCUMENT TG/201/1)

Document prepared by the Office of the Union

1. This document contains the presentations received during the forty-fourth session of the Technical Working Party for Fruit Crops, with regard to the partial revision of the Test Guidelines for Mandarin (TG/201/1).

2. The presentations from the European Union, Morocco, Spain and South Africa, are set out in the Annexes to this document.

[Annexes follow]

CPVO
Community Plant Variety Office

Proposal for a partial revision of the test guidelines for mandarin (citrus, Group 1)

TWF, Napier, 30 April 2013

Jean Maison

CPVO
Community Plant Variety Office

The Situation

Revision of the mandarin guideline

- Mandarin test guidelines were adopted in 2003
- Since then, problems occurred in varieties supposed to be seedless with the presence of seeds derived from unwanted cross pollination with other neighboring plantations

Revision of the mandarin guideline

- Breeding programs have developed in order to improve the situation with 2 kinds of methodologies:
  - triploid varieties (sterile pollen)
  - irradiation of varieties to reduce their fertility.

    Irradiated varieties have a reduced ovule fertility and produce no seeds independently from pollinators that might be present in the vicinity

Revision of the mandarin guideline

- Proposal for a partial revision of the test guideline in order to address these new types:
  - update characteristic 25 '*Anther viable pollen*', currently with two states of expression, 'Absent' – 'Present' where differences observed in the pollen germination percentage cannot be reflected with only two states. Proposal to move to a quantitative characteristic.
  - Proposal not contested

Revision of the mandarin guideline

- Proposal for a partial revision of the test guideline in order to address these new types:
  - to introduce a new characteristic to evaluate the ovule fertility by cross pollination with another variety or species: '*Fruit: number of seeds (controlled manual cross-pollination)*'
  - Proposal that needs further investigations

Revision of the mandarin guideline

- The TWF agreed in 2011 that
  - It should be checked whether this characteristic is eligible as a UPOV characteristic
  - The methodology for observing this characteristic should be well defined and tested
  - The wording of the characteristic might need to be reviewed

Revision of the mandarin guideline

- The TWF designed a subgroup
  - Morocco
  - South Africa
  - Spain
  - Coordination by the CPVO

Revision of the mandarin guideline

- The subgroup agreed on a protocol drafted on the basis of the Spanish proposal and after comments of other subgroup members in order to test the methodology for the assessment of the characteristic in question

- Work in the field started in March 2012

CPVO
Community Plant Variety Office

About the presence of seeds in Citrus varieties

Revision of the mandarin guideline

Incompatibility issues in Citrus are recognized to be gamete related: they are based on the unsuccessful growth of pollen tubes because the S allele carried by the haploid male pollen matches either of the two S alleles existing in the diploid maternal tissue of the pistil. It should be specified that clementines are self incompatible.

Revision of the mandarin guideline

- Consequence: the observation of the absence or the presence of few seeds for a variety can have different reasons (or combinations thereof)

1. The pollen of the pollinator is not viable
2. The pollen of the pollinator is incompatible with the stigma of the candidate variety
3. Ovules of the candidate variety are in limited numbers or have a reduced fertility.

Revision of the mandarin guideline

- It is important to define precisely what is intended to be observed and to define precisely the method of observation
  - Otherwise, a characteristic like 'Fruit : Number of seeds' might be the subject of very different observations depending on the pollination environment, might thus not be repeatable and finally not fulfill the definition of a UPOV characteristic.

Revision of the mandarin guideline

- Objective of the characteristic: to assess the ability of the variety to produce seeds under optimal fertilisation conditions
  - the pollinator and receiver should be fully compatible
    - No publication about groups of compatibility in Citrus
    - Molecular techniques to assess the compatibility of 2 Citrus varieties do not seem to be available
  - It should be make sure that the absence of seeds is linked to weak ovule fertility and/or their limited number and not to an incompatibility between the pollinator and the candidate variety

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Community Plant Variety Office

The ring test proposal

Revision of the mandarin guideline

- Proposal that the number of seeds is observed:
  - in counting the maximum number of seeds obtained after pollination with a range of good pollinators
  - under well described agroclimatic and pollen donor conditions
  - the pollination is made each time with pollen of a single pollinator rather than with a pollen mixture
    - The composition of which could be the subject of variation

Revision of the mandarin guideline

- For the purpose of the ring test, identification of varieties to be used as pollinators (male) and as receptors (female) available for all partners
  - Possibility to draw a pollination table
  - Comparison of the consistency of results
  - At the end of the ring test, conclusion whether the characteristic can be reliably assessed or not

Revision of the mandarin guideline

- The pollination table

		POLLINATOR	
		'Nova'	**'Nadorcott'**
Female	**'Marisol'**		
	'Clemenules' = 'Nules'		
	'Nadorcott'		
	'Nova'		

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Community Plant Variety Office

The results

Revision of the mandarin guideline

- The pollination table

		ES, First date		ES, second date		MO, Gharb		MO, Haouz		ZA, First date		ZA, second date	
		'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'
Female	'Marisol'	10,6	13,76	11,42	13,36	6	9	9	13	11,1	12,5	6,8	8,1
	'Clemenules'	22.32	29,44	19,2	29,28	16	29	17	23	23,9	27,5	8,4	9,9
	'Nadorcott'	13.96		14,16									
	'Nova'		30,12		29,4						28,4		20,6

Revision of the mandarin guideline

- The pollination table

		ES, First date		ES, second date		MO, Gharb		MO, Haouz		ZA, First date		ZA, second date	
		'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'
Female	'Marisol'	10,6	13,76	11,42	13,36	6	9	9	13	11,1	12,5	6,8	8,1
	'Clemenules'	22.32	29,44	19,2	29,28	16	29	17	23	23,9	27,5	8,4	9,9
	'Nadorcott'	13.96		14,16									
	'Nova'		30,12		29,4						28,4		20,6

➢ 'Nadorcott' is always the best pollinator

Revision of the mandarin guideline

- The pollination table

		Nova'						Nadorcott'					
		ES, First date	ES, second date	MO, Gharb	MO, Haouz	ZA, first date	ZA, second date	ES, First date	ES, second date	MO, Gharb	MO, Haouz	ZA, first date	ZA, second date
Female	'Marisol'	10,6	11,42	6	9	11,1	6,8	13,76	13,36	9	13	12,5	8,1
	'Clemenules'	22.32	19,2	16	17	23,9	8,4	29,44	29,28	29	23	27,5	9,9
	'Nadorcott'	13.96	14,16										
	'Nova'							30,12	29,4			28,4	20,6

➢ The number of seeds produced is quite consistent, abnormal results to be considered

Revision of the mandarin guideline

- The pollination table

		ES, First date		ES, second date		MO, Gharb		MO, Haouz		ZA, First date		ZA, second date	
		'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'	'Nova'	'Nadorcott'
Female	'Marisol'	10,6	13,76	11,42	13,36	6	9	9	13	11,1	12,5	6,8	8,1
	'Clemenules'	22.32	29,44	19,2	29,28	16	29	17	23	23,9	27,5	8,4	9,9
	'Nadorcott'	13.96		14,16									
	'Nova'		30,12		29,4						28,4		20,6

Lowest Number of seeds

Intermediate' number

Highest number

- The ranking of varieties seems to be quite consistent so far

Revision of the mandarin guideline

- The TWF agreed in 2011 that

- It should be checked whether this characteristic is eligible as a UPOV characteristic
  - The methodology for observing this characteristic should be well defined and tested
  - The wording of the characteristic might need to be reviewed

Revision of the mandarin guideline

- Verification that the characteristic fulfills the definition of a UPOV characteristic as laid down in section 4.2.1. of document TG/1/3

  ✓ The characteristic results from a certain genotype

  Under the conditions of the protocol

  ➢ The number of seeds depends on the number of ovules and their fertility. These characteristics have a genetic background.

Revision of the mandarin guideline

- Verification that the characteristic fulfills the definition of a UPOV characteristic as laid down in section 4.2.1. of document TG/1/3

  ✓ The characteristic is sufficiently consistent and repeatable in a particular environment

  Under the conditions of the protocol,

  ➢ Results obtained so far indicate that the characteristic is reproducible in different environments as the ranking of varieties remains the same

Revision of the mandarin guideline

- Verification that the characteristic fulfills the definition of a UPOV characteristic as laid down in section 4.2.1. of document TG/1/3

✓ Exhibits sufficient variation between varieties to be able to establish distinctness

Under the conditions of the protocol,

➢ The ring test clearly indicates consistent and sufficient significant variation between varieties

Revision of the mandarin guideline

- Verification that the characteristic fulfills the definition of a UPOV characteristic as laid down in section 4.2.1. of document TG/1/3

✓ Is capable of precise definition and recognition

A draft methodology has been defined for the ring test with at some stage different options.

➢ A precise definition of the characteristic and the methodology of observations are proposed later in this document

Revision of the mandarin guideline

- Verification that the characteristic fulfills the definition of a UPOV characteristic as laid down in section 4.2.1. of document TG/1/3

  ✓ Allows uniformity requirements to be fulfilled

    ➢ This could be assumed as this is a vegetatively propagated species

Revision of the mandarin guideline

- Verification that the characteristic fulfills the definition of a UPOV characteristic as laid down in section 4.2.1. of document TG/1/3

  ✓ allows stability requirements to be fulfilled

    ➢ Considering that the trees representing varieties are issued at different places of the ring test from a different generation in the phase of propagation, it can be concluded that stability requirements can be fulfilled.

Revision of the mandarin guideline

- The TWF agreed in 2011 that
  - It should be checked whether this characteristic is eligible as a UPOV characteristic

➢ The methodology for observing this characteristic should be well defined

  - The wording of the characteristic might need to be reviewed

Revision of the mandarin guideline

- Hand pollination
- Conditions of the Pollen of the Donor
  - Pollen from male varieties will be at its optimal fertility

    The pollen of the pollinator variety is extracted at the moment of maximum development of the flower, before the anther dehiscence, detailed methodology defined in the protocol
  - Viability : germination capacity at least 50%, assessment after pollen culture, detailed methodology is defined in the protocol
  - Should this still be systematically checked?

Revision of the mandarin guideline

- Conditions of the Female flowers
  - Trees will be at the phenologic state of full bloom
  - Flowers will be fully developed but not yet opened: one day before anthesis
  - 50 terminal flowers from each female variety will be used, if possible 10 flowers from 5 trees. If less than 5 trees, the number of trees to be pollinated should be specified
  - The standard deviation after the ring test suggests that this number is necessary on order to characterize significant differences

Revision of the mandarin guideline

- Agroclimatic conditions
  - Wet days are not suitable and the temperature should be above 20˚

Revision of the mandarin guideline

- Conditions of the Female flowers
  - stigma of female varieties will be at its optimum stage of receptivity. Flowers will be cross-pollinated by hand respectively 1 to 6 days after anthesis
  - The ring test indicates that 1 day after anthesis gives clearly better results in MO & ZA, little difference in ES
  - Flowers will be emasculated and bagged to avoid self and free pollination

Revision of the mandarin guideline

- Methodology of Hand pollination
  - Pollination is made each time with pollen of a single pollinator
  - Proposal that 'Nadorcott' is the pollinator retained
  - Pollination should take place at the moment of maximum development of the flower
  - Quantity of pollen : saturation with a brush

Revision of the mandarin guideline

- After pollination
  - After pollination, the pollinated flowers should be isolated from the environment with an individual mesh in order to avoid further pollination
  - If felt necessary, 48 hours after pollination, the germination of pollen on stigma will be checked, detailed methodology described in the protocol

Revision of the mandarin guideline

- Observation of results
  - Fruits produced from manual pollination should be identified
  - Seeds produced in each individual resulting fruit must be counted and registered when the fruits are at stage of fruit maturity or one month before to get an average of the number of seeds per fruit. The number of fruits observed for each variety should be reported.
  - What about abortive and small/triploid seeds ?

Revision of the mandarin guideline

- The TWF agreed in 2011 that
  - ➢ It should be checked whether this characteristic is eligible as a UPOV characteristic
  - ➢ The methodology for observing this characteristic should be well defined

➢ The wording of the characteristic might need to be reviewed

Revision of the mandarin guideline

- Wording proposed

'*Fruit: number of seeds (controlled manual cross-pollination)*'

Does it need to be reviewed?

Revision of the mandarin guideline

- Some other issues

The following characteristic might need to be removed from the current guidlines:

99.		**Fruit: number of seeds (open pollination)**	**Fruit: nombre de pépins (fécondation libre)**	**Frucht: Anzahl Samen (frei abblühend)**	**Fruto: número de semillas (polinización libre)**		
QN (+)	(c)	absent or very few	absents ou très peu nombreux	fehlend oder sehr gering	ausentes o muy bajo	Clementules (CLE)	1
		few	peu nombreux	gering	bajo	Ellendale (TNR)	3
		medium	moyen	mittel	medio		5
[326]		many	nombreux	groß	alto	Común (MMN)	7

Revision of the mandarin guideline

- Conclusion
  - Does the variety fulfill the definition of a UPOV characteristic?
  - Can the methodology be adopted?
  - Should this characteristic be part of the guidelines or could it be an additional characteritic?
  - Can the wording for char 25 be proposed and char 99 be deleted?


Thank you for your attention

Report of the results of the study conducted in Morocco relating to variability in the number of seeds in the mandarin tree group in manual pollination conditions

Following the decisions of the Technical Working Party for Fruit Crops concerning the conduct of a study on the fertility of the ovule in certain varieties of citrus fruits through the estimation of the number of seed produced in manual pollination conditions, an experimental protocol has been prepared in consultation between Morocco, Spain and South Africa, and coordinated by the European Union.

The pollination tests are carried out by the three countries involved in the sub-group: Morocco, Spain and South Africa.

This report includes:

- **details of the experimental protocol adopted;**
- **the main results obtained;**
- **the conclusions drawn from the results.**

Overall aim

The study of the fertility of the ovule in a number of varieties of mandarin tree through the estimation of the number of seed produced in manual pollination conditions.

Specific aims

- **Estimation of the percentage of *in vitro* germination of pollen grains.**
- **Estimation of the receptivity of the female variety stigma through the estimation of the in vivo germination of pollen on stigmat after pollination**
- **Estimation of the number of seed produced in manual pollination conditions based on the pollination date, the pollinator male, the concentration of the pollen grain used and the region.**
- **Estimation of the number of seed in open pollination conditions.**

Details of the experimental protocol

1. Region

The study was conducted in Morocco according to the protocol adopted by the countries participating in the ring test. The experiment was carried out in two regions: Al Gharb, a subhumid region located near the coast, and Al Haouz, an arid continental region. The tests were conducted on trees of 10 to 15 years old.

2. Pollinators and females used

The varieties used in the experiment are shown in Table 1

Table 1: Male and female varieties used

		POLLINATOR	
		'Nova'	'Nadorcott'
Female	'Marisol'		
	'Clemenules' = 'Nules'		
	'Nadorcott'		
	'Nova'		

• Conditions of the donor pollen

- The pollen of the pollinator variety is extracted at the moment of maximum development of the flower, before the anther dehiscence.
- Open flowers at a growth stage of 61–62 on the Citrus BBCH phenological scale [Agusti *et al*., 1997] will be randomly selected for pollen collection at least the day before pollination.
- Anthers are put in petri dishes at room temperature with a dehydrator (e.g. a piece of paper) until they open.

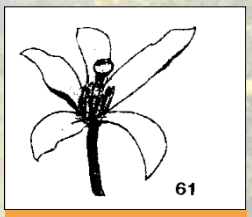


Photo: Agusti *et al*., 1997]

Stage of 61–62 on the Citrus BBCH phenological scale

Photo: INRA-ONSSA, 2012

Flowers at stage of 61–62 on the Citrus BBCH phenological scale

Photo: INRA-ONSSA, 2012

Anthers of donors at room temperature

Pollen viability

- Verification of the germination capacity of the pollen and utilization of pollen with at least 50% germination capacity (assessment after pollen culture).
- The pollen should be collected when the petals begin to open (but with the anthers closed).
- The anthers should be introduced into a petri dish and placed inside a silica gel dryer at room temperature, for 20-48 hours of darkness.
- When the anthers are open they should be moved to an 8ºC chamber with 70-80% Relative Humidity (RH) for one hour. Afterwards, the pollen should be brushed onto a microscope slide with 2 ml of Brewbaker medium (Brewbaker and Kwack, 1963). Finally, the microscope slide should be placed in a 24ºC chamber with 75% RH for 20 hours.
- The percentage of pollen fertilization is calculated as the average of germinated pollen grains observed with binoculars in 15 visual fields from two different microscope slides. Pollen from male varieties will be at its optimal fertility level.

Photo: INRA-ONSSA, 2012

Pollen of donors

Photo: INRA-ONSSA, 2012

Pollen incubation in Brewbaker medium

Photo: INRA-ONSSA, 2012

Photo: INRA-ONSSA, 2012

Germinated pollen grains observed with binoculars

Conditions of female flowers

- 50 terminal flowers from each female variety will be used, if possible 10 flowers from five trees.
- Flowers will be fully developed but not yet opened: one day before anthesis (stage 59 on the Citrus BBCH phenological scale [Agusti *et al.*, 1997]), flowers will be emasculated and bagged to avoid self and free pollination.
- The stigma of female varieties will be at its optimum stage of receptivity. Alternatively, 50 flowers a day will be cross-pollinated by hand one and six days respectively after anthesis.
- Other flowers at the same stage (stage 59) at the time of emasculation must be marked to indicate the number of days after anthesis and to mark the flowers emasculated at the time of anthesis (see additional explanation in Annex). Trees will be at the phenological state of full bloom.

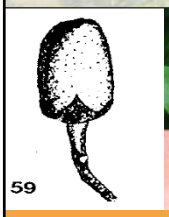


Photo: Agusti *et al.*, 1997]

Stage 59 on the Citrus BBCH phenological scale

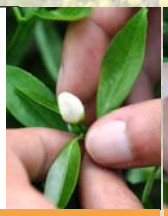


Photo: INRA-ONSSA, 2012


Photo: INRA-ONSSA, 2012

Emasculated flowers

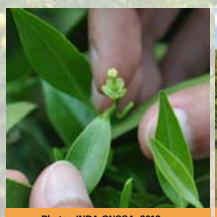


Photo: INRA-ONSSA, 2012


Photo: INRA-ONSSA, 2012

Bagged flowers to avoid self and free pollination

Hand pollination

- Pollination is made each time with the pollen of a single pollinator.
- Pollination should take place at the moment of maximum development of the flower.
- Quantity of pollen: to reach the saturation of the stigma, at least 100 grains of pollen.
- Dissecting microscope is used in the field to evaluate the quantity of pollen grains, which must be more than 100. Those flowers will be compared with the one with massive amounts of fresh pollen.
- Pollen will be applied with a brush.
- After pollination, the pollinated flowers should be isolated from the environment with an individual mesh in order to avoid further pollination.


Photo: INRA-ONSSA, 2012

Dissecting microscope is used in the field to evaluate the quantity of pollen grains.


Photo: INRA-ONSSA, 2012

Pollen to be applied with a brush


Photo: INRA-ONSSA, 2012

Pollinated flowers


Photo: INRA-ONSSA, 2012

Pollinated flowers isolated from the environment by a person

Stigma receptivity

- For each pollinating date, 10 additional flowers per variety will be removed 48 hours 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 grains per pollinated flower.
- A pollen grain was considered to be germinated when the pollen tube length was longer than the pollen grain diameter. This is done by extracting the germinated pollen grain in the laboratory using the microscope.

Photo: INRA-ONSSA, 2012

Stigma fixation in FPA

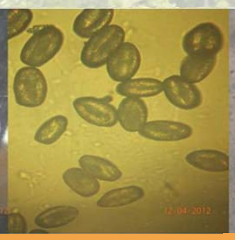


Photo: INRA-ONSSA, 2012

Non-germinated pollen *in vivo* (in the stigma after pollination)

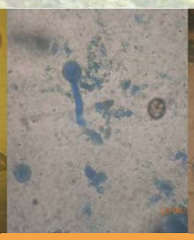


Photo: INRA-ONSSA, 2012

Photo: INRA-ONSSA, 2012

Germinated pollen in the stigma after pollination

PRELIMINARY RESULTS
First year

I- Percentage of *in vitro* germination of pollen grains for Nadorcott and Nova mandarin varieties

Figures 1 and 2 show the *in vitro* germination of the pollen grains of the two male varieties used: Nadorcott mandarin and Nova mandarin.

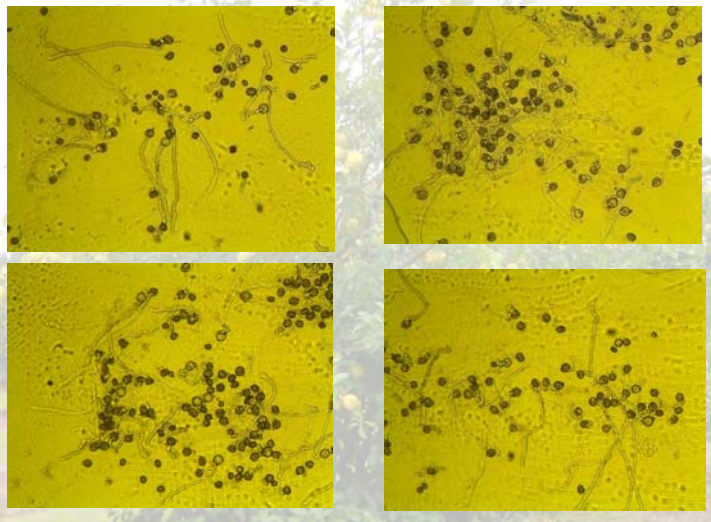


Figure 1 : *In vitro* germination of pollen grains of the Nadorcott mandarin variety

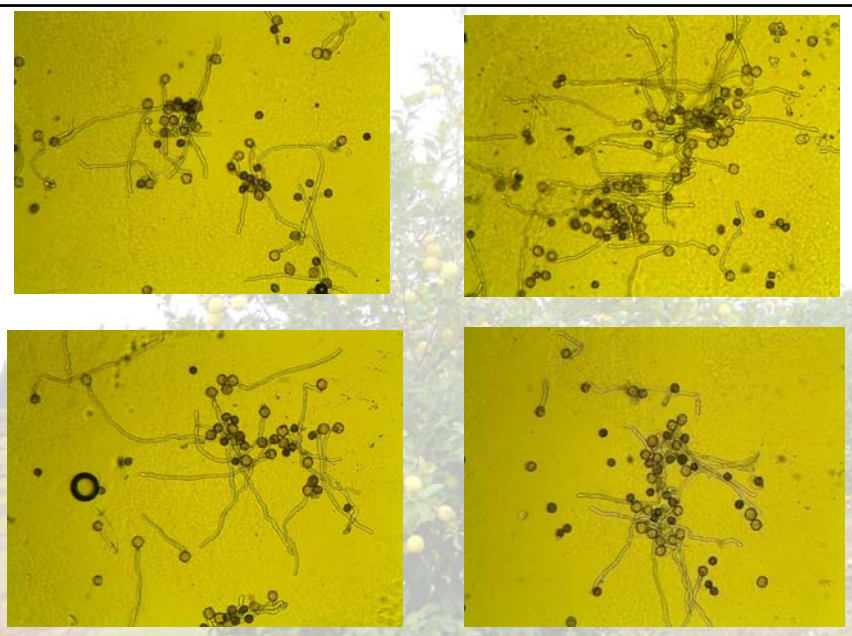


Figure 2: *In vitro* germination of pollen grains of the Nova mandarin variety

Figure 3 shows that in an agar medium, the pollen grains of the two varieties used recorded germination percentages of 68.83% et 67.17% respectively for the Nova mandarin and the Nadorcott mandarin.

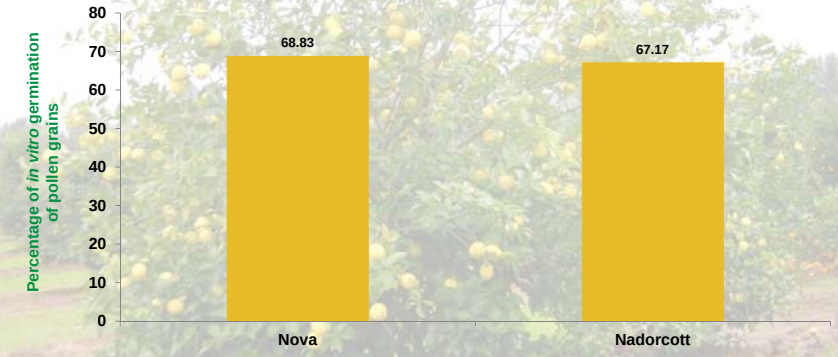


Figure 3: Percentage of *in vitro* germination of pollen grains of the Nova mandarin and Nadorcott mandarin varieties

II- Receptivity of stigmata

The receptivity of the stigma of a variety is expressed by the percentage of *in vivo* germination, in relation to the stigma, and of the pollen grain of the male variety, one and six days after the anthesis.

Figure 4 shows that the percentage of germination of the pollen grains of the Nadorcott mandarin for the stigmata of the female varieties used underwent a reduction after six days of the anthesis. Furthermore, a comparison of the average percentages of germination of the pollen grains for the stigmata, both one day and also six days after anthesis, shows that the pollen grains of the Nadorcott mandarin behave differently in terms of percentage of germination for the stigmata of the female varieties used. Indeed:

- **One day after anthesis:** The highest percentage of germination of the pollen grains of the Nadorcott mandarin is recorded for the stigma of the Nules clementine, i.e. 26.38%. Moreover, the lowest percentage of germination of pollen grains is recorded for the stigma of the Nadorcott mandarin variety, i.e. 14.62%.
- **Six days after anthesis**: The lowest percentage of germination of the pollen grains of the Nadorcott mandarin is recorded for the stigma of the Nadorcott mandarin variety (10.51%) against 20.73%, 20.9% and 18.47% respectively for the stigmata of the Nules clementine, Nova mandarin and Marisol clementine.

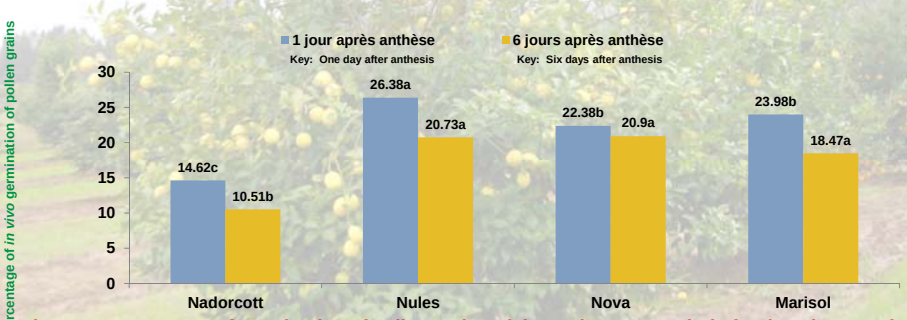


Figure 4: Percentage of germination of pollen grains of the Nadorcott mandarin for the stigmata of four varieties, one day and six days after anthesis.

For the same date, the varieties followed by the same letter do not differ signicantly at the five per cent threshold (Duncan Test)

Figure 5 shows that for the four varieties used, the percentage of germination of the pollen grains of the Nova mandarin for the stigmata underwent a reduction after six days of the anthesis. Furthermore, a comparison of the average percentages of germination of the pollen grains for the stigmata, both one day and also six days after anthesis, shows that the pollen grains of the Nova mandarin behave differently in terms of percentage of germination based on the female variety used. Indeed:

- **One day after anthesis**: The highest percentage of germination of pollen grains is recorded for the stigma of the Marisol clementine, i.e. 32.25%. By contrast, the lowest percentage is recorded for the stigmata of the Nadorcott mandarin, Nules clementine and Nova mandarin. The values are 16.69%, 17.83% and 16.37 % respectively.
- **Six days after anthesis**: The lowest percentage of germination of pollen grains is recorded for the stigmata of the Marisol clementine (10.24%) and Nadorcott mandarin (9.7%). This percentage was 15.89% for the Nules and 12.64% for the Nova respectively.

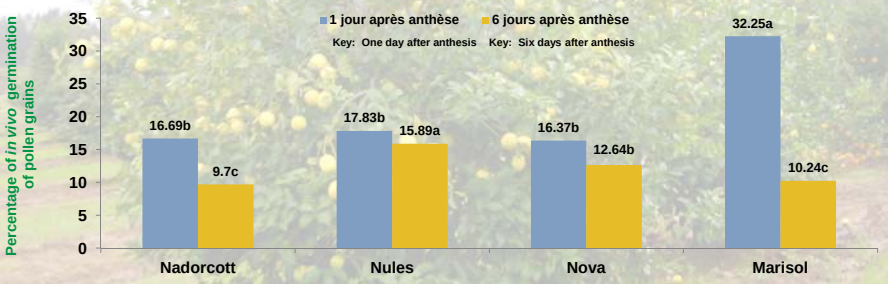


Figure 5: Percentage of germination of pollen grains of the Nova mandarin for the stigmata of four varieties: one and six days after anthesis

For the same date, the varieties followed by the same letter do not differ sigifcantly at the five per cent threshold (Duncan Test)

Figure 6 shows that the percentage of germination of the pollen grains of the Nadorcott mandarin for the stigmata of the female varieties used underwent a reduction after six days of the anthesis. Furthermore, a comparison of the average percentages of germination of the pollen grains for the stigmata, both one day and also six days after anthesis, shows that the pollen grains of the Nadorcott mandarin behave differently in terms of percentage of germination for the stigmata of the female varieties used. Indeed:

- **One day after anthesis:** The highest percentage of germination of the pollen grains of the Nadorcott mandarin is recorded for the stigma of the Nova mandarin, the Marisol Clementine and the Nules Clementine, which are 26.44%, 25.8% and 22.7% respectively. By contrast, the lowest percentage of germination of the pollen grains is recorded for the stigma of the Nadorcott mandarin variety, i.e. 13.7%.

- **Six days after anthesis**: The lowest percentage of germination of the pollen grains of the Nadorcott mandarin is recorded for the stigma of the Nadorcott mandarin variety (12.36%) against 25.48%, 24.4% and 23.9% respectively for the stigmata of the Marisol clementine, Nova mandarin and the Nules clementine.

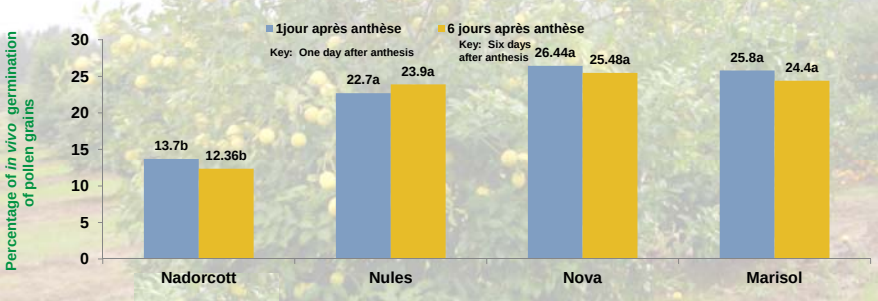


Figure 6: Percentage of germination of pollen grains of the Nadorcott mandarin for the stigmata of four varieties, one and six days after anthesis.

For the same date, the varieties followed by the same letter do not differ significantly at the five per cent threshold (Duncan Test)

Figure 7 shows that for the four varieties used, the percentage of germination of the pollen grains of the Nova mandarin for the stigmata underwent a reduction after six days of the anthesis. Furthermore, a comparison of the average percentages of germination of the pollen grains for the stigmata, both one day and also six days after anthesis, shows that the pollen grains of the Nova mandarin behave differently in terms of percentage of germination depending on the female variety used. Indeed:

- **One day after anthesis**: The highest percentage of germination of the pollen grains is recorded for the stigma of the Nules clementine (25.17%), the Marisol clementine (23.2%) and the Nadorcott mandarin (23.18%). By contrast, the lowest percentage of germination of the pollen grains is recorded for the stigmata of the Nova mandarin, i.e. 11.9%.
- **Six days after anthesis**: The lowest percentage of germination of the pollen grains is recorded for the stigmata of the Nova mandarin (10.5%) against 23.11%, 22.4% and 22.2% respectively for the Nadorcott mandarin, the Nules clementine and Marisol clementine.

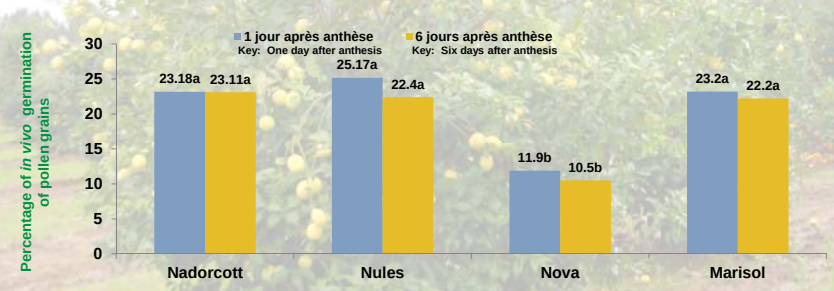
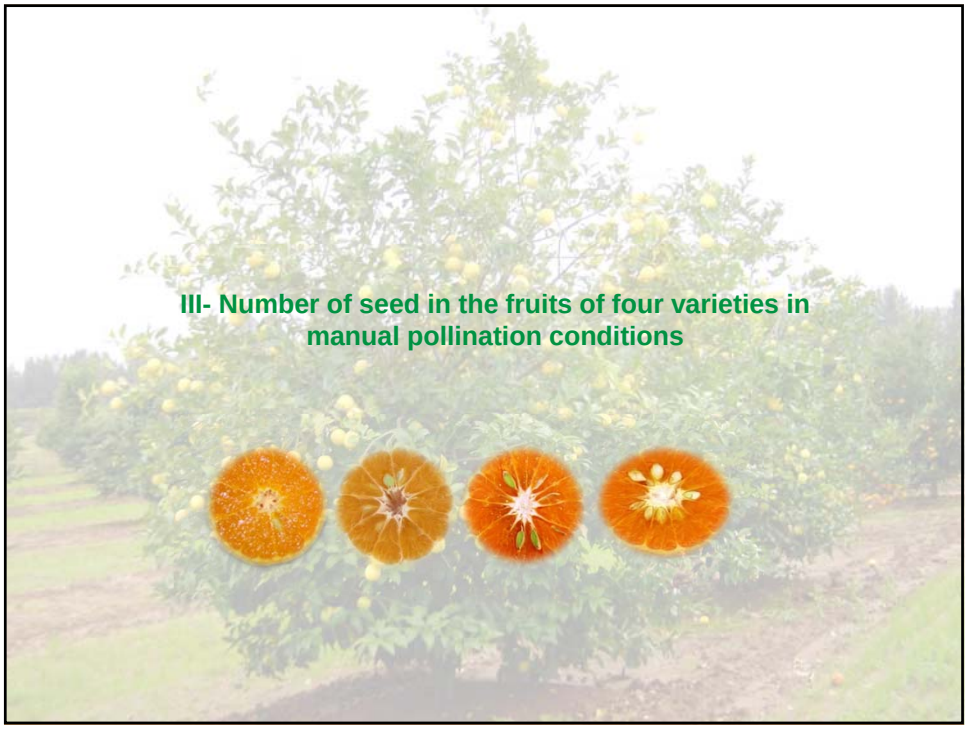


Figure 7: Percentage of germination of pollen grains of the Nova mandarin for the stigmata of four varieties, one and six days after anthesis.

For the same date, the varieties followed by the same letter do not differ significantly at the five per cent threshold (Duncan Test)



The heat conditions which were rife in Morocco during the setting period caused significant falls of young fruit, in particular in the Nova*Nova, Nadorcott*Nadorcott and Nadorcott*Nova cross breeds.
The ANOVA test therefore concerned only two female varieties, i.e. NULES and MARISOL.

Thus, an analysis of the variance in the number of seed recorded for the fruit of two varieties in manual pollination conditions (Table 10) shows a very highly significant effect of the male variety, the pollination date, the concentration of pollen and the female variety used. Similarly, significant interactions were recorded for the factors: Site*Date, Male*Date, Female variety*Date, and Female variety*Male variety.
The number of seed for the fruit is therefore influenced by several factors: male variety, female variety, concentration of pollen grains used.

Table 10: Summary table of the analysis of the variance in the number of pips recorded in two varieties of mandarin tree

Source	DL	F Value	Pr > F
Site	1	0.91	0.3436
Male	1	56.98	<.0001
Site*Male	1	1.81	0.1829
Date	1	87.54	<.0001
Site*Date	1	31.84	<.0001
Male*Date	1	5.16	0.0258
Concentration	1	35.24	<.0001
Site*Concentration	1	0.73	0.3951
Male*Concentration	1	1.43	0.2348
Date*Concentration	1	0.00	0.9806
Variety	1	190.50	<.0001
Site*Variety	1	16.64	0.0001
Male*Variety	1	11.53	0.0011
Date*Variety	1	38.20	<.0001
Concentration*Var	1	1.32	0.2543

Table 11 shows that for the two regions (Al Gharb and Al Haouz), the number of seed recorded in the Nules clementine varies depending on the male parent, the date of pollination and the concentration of the pollen grain used. The highest number of seed is recorded in the presence of the Nadorcott as male parent, for pollination one day after anthesis and saturated concentration of the pollen grain.

Table 11: Number of seeds recorded in the Nules clementine variety for the two sites based on the male pollinator, the date of pollination and the concentration of the pollen grain.
For the same line, the males, dates and concentrations, and the figures followed by the same letter do not differ significantly at the five per cent threshold (Duncan Test)

Variety	Site	Male		Date		Concentration	
		Nadorcott	Nova	1 day	6 days	Saturation	100g
Nules	Site 1: Al Gharb	29a	16b	25 a	20 b	24 a	21 b
	Site 2: Al Haouz	23a	17 b	30a	9b	23a	17 b

Table 12 also shows that for the two regions (Al Gharb and Al Haouz), the number of seed recorded in the Marisol clementine varies depending on the male parent, the pollination date and the concentration of the pollen grain used. The highest number of seed is recorded in the presence of the Nadorcott as male parent, for pollination one day after anthesis and saturated concentration of the pollen grain.

Table 12: Number of seed recorded in the Nules clementine variety for two sites depending on the male pollinator, the pollination date and the concentration of the pollen grain.
For the same line, the males, dates and concentrations, and the figures followed by the same letter do not differ significantly at the five per cent threshold (Duncan Test)

Variety	Site	Male		Date		Concentration	
		Nadorcott	Nova	1 day	6 days	Saturation	100g
Marisol	Site 1: Al Gharb	9a	6b	8 a	6b	10a	4b
	Site 2: Al Mar	13a	9 b	13 a	9 b	14 a	8 b

IV- Number of seed in fruits in open pollination conditions

Figure 8 shows that the number of seed for fruit, in open pollination conditions, varies between the varieties used. Indeed, a comparison of the average numbers of seed recorded in the varieties allowed four statistically separate groups to be identified at the five per cent threshold (Duncan Test).

The first group is represented by the Nules clementine variety. This variety recorded the highest number of seed for fruit, a total of 14.88 seed.
The second group is represented by the Nadorcott mandarin variety with 12.35 seed per fruit.
The third group is represented by the Marisol clementine variety (5.56 seed).
The fourth group is represented by the Nova mandarin (2.35 seed).

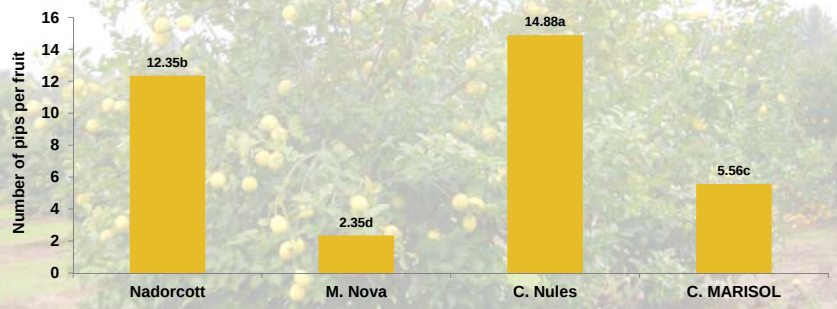


Figure 8: Number of pips recorded in four varieties of citrus fruits in open pollination conditions
The varieties followed by the same letter do not differ significantly at the five per cent threshold (Duncan Test)

Discussions

The results obtained in this work demonstrate that the fertility of the ovule in varieties of citrus fruits is complex in nature, and is influenced by several factors: the pollinator parent, the female variety, the receptivity of the stigma, the pollination date the region and the concentration of the pollen grains used.

In our study we have shown that the receptivity of the stigma in the female varieties used, which is estimated by the percentage of *in vivo* germination of the pollen grains, decreases the further away you move from the anthesis stage. Moreover, the varieties showed a difference in terms of receptivity, expressed by the percentage of *in vivo* germination of the pollen grain of the male parent. The results we obtained are consistent with the work done by Mesejo *et al*.,2007, who noted that the Owari satsuma variety has a short period of receptivity compared to the Valencia Late and Nules clementine varieties. The difference between the periods of receptivity of the stigmata has also been reported between different species of the Pyrus and Malus genus by Sanzol and Herrero (2001).

Concerning the effect of the pollination date on the number of seed in the varieties used, in this study we have shown that the number of seed decreases for pollination carried out six days after anthesis. In this sense, Mesejo *et al*. (2007) noted that the number of seed obtained in varieties of citrus fruits varies depending on the stage of pollination. The authors noted in the Nules clementine a reduction in the number of seed based on the number of days after the anthesis stage.

In terms of concentration of the pollen grains used for pollination, the results of this study helped to identify an increase in the number of seed with an increase in the concentration of the pollen grains used. The results obtained are consistent with the work of Brown and Krezdorn (1969). These authors showed that the number of seed obtained for a concentration of 50 pollen grains is significantly lower than that obtained for a concentration of 100 pollen grains.

The use of open pollination in this study allowed a significant distinction to be drawn in the degree of fertility of the ovule of the varieties used on the basis of the number of seed produced by each variety. Our results in this regard are consistent with the work of Masashi *et al*. (1995) who demonstrated the importance of open pollination in the determination of the fertility of the ovule in citrus fruits. This technique is not influenced either by the concentration of the pollen grain or by the physiological stage of pollination.

Conclusions

On the basis of the results obtained in this study we can conclude that:

- the receptivity of the stigma, expressed in terms of percentage of *in vivo* germination of the pollen grains, varies depending on the site, pollination date and female variety.

- for a single variety, the number of seed varies depending on the site, the pollinator male, the concentration of the pollen grain and the pollination date.

- in open pollination conditions, the varieties studied are divided into four statistically separate groups according to the number of seed per fruit produced by each variety.

- the heat conditions which were rife in Morocco during the setting period caused significant falls of young fruit, in particular in the Nova*NOV, Nadorcott*Nadorcott and Nadorcott*Nova cross breeds.

The results obtained during the first year allowed a clear distinction to be drawn between the varieties in terms of feritility of the ovule expressed by the number of seed produced in open pollination conditions, whereas in manual pollination conditions the degree of fertility is strongly influenced by the origin of the pollen, the region, the concentration and the pollination date.

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44º TWF- Napier (NZ)

UPOV MANDARINE RING TEST

NEW CHARACTERISTIC TO EVALUATE THE OVULE FERTILITY BY CONTROLLED MANUAL CROSS-POLLINATION

RESULTS FROM SPAIN

OEVV (SPANISH PLANT VARIETY OFFICE)
UETIV (TECHNICAL EXAMINATION UNIT FOR VARIETY IDENTIFICATION)
•Guillermo Soler
•Aurelio Buj

April 22, 2013

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THE OBJECTIVE OF THE RING TRIAL

• **The CPVO ring test proposal says:** *The objective of this ring trial is to reach an agreement on such conditions in order to have a reliable observation of the characteristic. (the number of seeds after manual cross-pollination can only reflect properly the ovule fertility if a set of conditions is met)*

• **The 42th TWF reached the following conclusion (TWF 42/26 "repport"): ....** *The TWF discussed the proposed mandarin partial revision and agreed that further studies were necessary* ***to test the methodology****.*

RING TEST OBJECTIVE: TO SET THE CONDITIONS OF METHODOLOGY

(for the new characteristic)

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METHODOLOGY

• **Conditions of the pollen of the donor:**

***Viability* : Verification of the *germination capacity* of the pollen and utilization of pollen with *at least 50% germination* capacity (assessment after pollen culture).**

Method: *The pollen should be collected when the petals begin to open (but with the anthers closed). The anthers should be introduced into a Petri dish and placed inside a silica gel dryer at room temperature, for20-48 hours of darkness. When the anthers are open they should be moved to a 8 ºC chamber with a 70-80 % Relative Humidity for one hour. Afterwards, the pollen should be brushed onto a microscope slide with 2 ml of Brewbacker medium (Brewbaker and Kwack. 1963). Finally, the microscope slide should be placed in a 24 ºC chamber with a 75 % RH for 20 hours.*
The percentage of pollen fertilization is calculated as the average of germinated pollen grains observed with a binocular in 15 visual fields from 2 different microscope slides.
(Brewbaker, J.L. and Kwack, B.H. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. Amer. Jour. Botany. 50: 859-865.)

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METHODOLOGY

Conditions of the pollen of the donor:

• ***Results* :**

Nadorcott

Nova

Pollen Germination Percentage			
SPAIN		MOROCCO	
NOVA	NADORCOTT	NOVA	NADORCOTT
57,00	61,90	68,83	67,17

• As required in the methodology in both cases the pollen viability used was above 50%.

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METHODOLOGY

• **Conditions of female flowers:**

Method:

- *50 terminal flowers from each female variety will be used, if possible 10 flowers from 5 trees. If less than 5 trees, the number of trees, the number of female trees to be pollinated should be specified*

- *Flowers will be fully developed but not yet opened: one day before anthesis (stage 59 on the Citrus BBCH phenological scale [Agust and al., 1997]), flowers will be emasculated and bagged to avoid self and free pollination.*

- *Stigma of female varieties will be at its optimum stage of receptivity. Alternatively, 50 flowers a day will be cross-pollinated by hand respectively 1 and 6 days after anthesis.*
  (THE PROPOSAL FROM SPAIN WAS TO FIX ONLY 1 DAY AS OPTIMUM STAGE OF RECEPTIVITY OF STIGMA, TO AVOID VARIABILITY)

- *Trees will be at the phenologic state of full bloom.*

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METHODOLOGY

• ***Date of pollination - Results :***

	Nº seeds/fruit	
	SPAIN	MOROCCO
	Significant difference	Significant difference
Date Pollination	NO	YES

• Despite not having significant differences in the date of pollination in Spain, the data from Morocco shows some differences but with pollination result better 1 day after anthesis.

SINCE THE AIM OF THE TRIAL IS TO FIX THE CONDITIONS OF METHODOLOGY, IT IS LOGICAL SET AS MOMENT OF POLLINATION 1 DAY AFTER ANTHESIS TO AVOID VARIABILITY

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METHODOLOGY

• **Hand pollination:**

Method:

• *Pollination is made each time with pollen of a single pollinator.*
  • *Pollination should take place at the moment of maximum development of the flower.*
  • *Quantity of pollen : to reach the saturation of the stigma, at least 100 grains of pollen*
  • *Pollen will be applied by a brush*
  • *After pollination, the pollinated flowers should be isolated from the environment with an individual mesh in order to avoid further pollination*

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METHODOLOGY

• ***Hand pollination -Results:***

• First consideration**: any study of pollen concentration on the stigma was completely unnecessary (Proposal says:** *Methodology of hand pollination; Quantity of pollen: to reach the saturation of the stigma*)

• Second consideration: **All data shows better results when the proposed methodology is followed strictly.**

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MAIN RESULTS OF THE RING TEST

NUMBER OF SEEDS PER FRUIT

SPAIN

		Nº seeds/fruit							
		MALE							
		NOVA				NADORCOTT			
		SPAIN				SPAIN			
FEMALE	MARISOL	10,60				13,76			
	CLEMENULES	22,32				29,44			

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MAIN RESULTS OF THE RING TEST

NUMBER OF SEEDS PER FRUIT

SPAIN AND MOROCCO

		Nº seeds/fruit							
		MALE							
		NOVA				NADORCOTT			
		SPAIN	MOROCCO Gharb	MOROCCO Haouz		SPAIN	MOROCCO Gharb	MOROCCO Haouz	
FEMALE	MARISOL	10,60	6,00	9,00		13,76	9,00	13,00	
	CLEMENULES	22,32	16,00	17,00		29,44	29,00	23,00	

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MAIN RESULTS OF THE RING TEST

NUMBER OF SEEDS PER FRUIT

SPAIN ,MOROCCO and SUDAFRICA

		Nº seeds/fruit							
		MALE							
		NOVA				NADORCOTT			
		SPAIN	MOROCCO Gharb	MOROCCO Haouz	SOUTH AFRICA	SPAIN	MOROCCO Gharb	MOROCCO Haouz	SOUTH AFRICA
FEMALE	MARISOL	10,60	6,00	9,00	8,10	13,76	9,00	13,00	9,20
	CLEMENULES	22,32	16,00	17,00	22,40	29,44	29,00	23,00	25,40

- **In all trials, independently of the good pollinator used the number of seeds per fruit in each female variety was very similar.**
- **In all trials, the Clemenules variety showed a number of seeds significantly higher than variety Marisol.**

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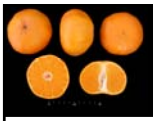
CONCLUSION

THESE RESULTS INDICATE THAT THE DESCRIBED METHODOLOGY (MANUAL CONTROLLED CROSS-POLLINATION) IS EFFECTIVE FOR THE DETERMINATION OF THE NUMBER OF SEEDS.



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COMPARED METHODOLOGY:

- **(*Controlled manual cross-pollination) versus (Open pollination):***

- **The described methodology fix the most of environmental factors:**

  - **Bees and other insects availability and their preferences: manual pollinatión.**

  - **Stigma pollination at its optimum stage of receptivity: 1 day after anthesis.**

  - **Quantity of pollen: to reach the saturation of the stigma.**

  - **Quallity of pollen: using well known good pollinators.**

CONCLUSION:
OPEN POLLINATION HAS UNCONTROLED FACTORS, MANUAL CROSS-POLLINATION IS A REPEATABLE AND CONFIDENT CHARACTER

RING TEST PROPOSAL

-

REPORT FROM SOUTH AFRICA

Regina Cronje[1], Christo Human[1], Nikki Combrink[2], Johan Maritz[2], Innocent Ratlapane[1], Carensa Petzer[3] and Mark Schaffner[3]

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agriculture, forestry & fisheries
Department: Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

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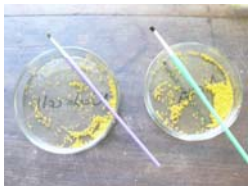
Excellence in Research and Development

Background

- Proposed partial revision of mandarin test guidelines
- Introduction of new characteristic to evaluate ovule fertility by controlled manual cross pollination with another variety or species: 'Fruit: number of seeds (controlled manual cross pollination)'
- Test methodology for new characteristic for suitability for test guidelines
- Countries collaborating in Ring Test are Spain, Morocco and South Africa

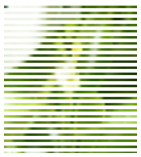
Materials & Methods

- Trial Sites:
  - Addo Research Station in Eastern Cape (for pollinations on Marisol, Nules, Nova)
  - Farm Sun Orange near Addo (for pollinations on Nadorcott)
  - Climate for both sites: temperate costal summer/winter rainfall area
- Pollen preparation as per methodology:
  - Pollen collection 2 days before pollination
  - Pollen germination test
  - Emasculation and flower bagging one day before anthesis


Materials & Methods cont.

- Pollinations :
  - At anthesis (3 October 2012)
  - 6 days after anthesis (9 October 2012)
  - 70 flowers per open, self and controlled manual cross pollination and pollination date
  - 48 hours after pollination: 10 flowers per cross pollination collected for in vivo pollen germination study


- Evaluations (April 2013):
  - Number of seed per fruit (full, small and abortive seed) for Marisol, Nules, Nova (Nadorcott not yet ready)
  - Fruit set for Marisol, Nules, Nova (Nadorcott not yet ready)
  - No statistical analysis done yet

RESULTS

Pollen germination test

- 50% pollen germination capacity as per guideline was not achieved

	Germinated pollen (%)	
Test date	**Nadorcott**	**Nova**
04-Oct-12	44	45
09-Oct-12	54	36

- Pollen viability (acetocarmine staining method) showed high pollen viability for male parents

	% Pollen viability
Nadorcott	73.2
Nova	84.3

- In vivo germination test is still underway

Number of fruit evaluated

- Controlled manual cross pollination showed an increased fruit set potential compared to open pollination

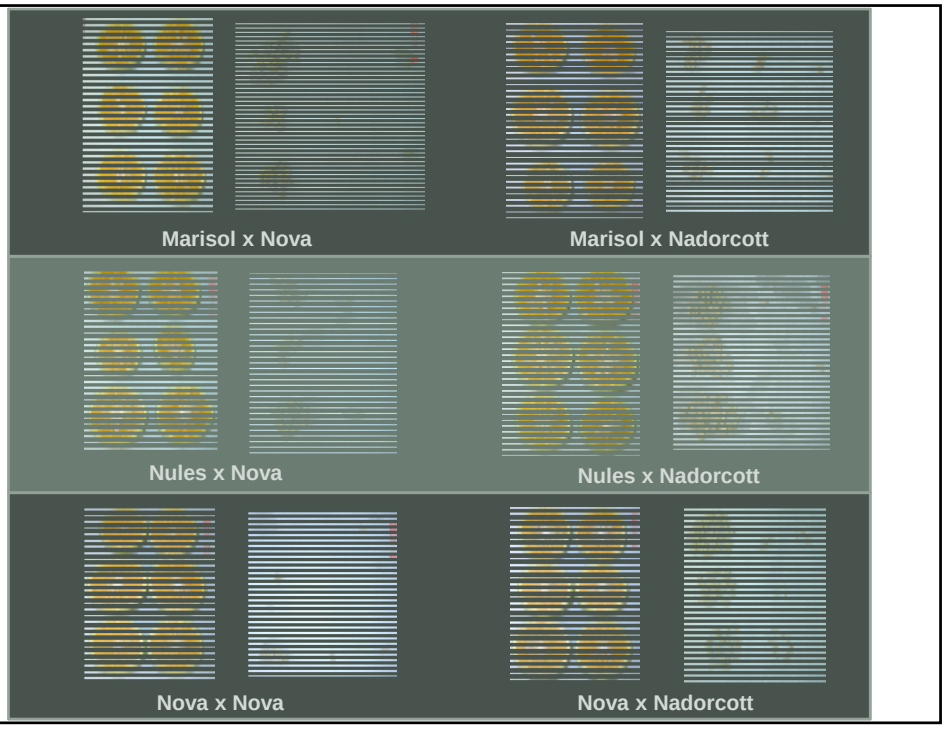
		No. of fruit harvested and evaluated from 70 pollinated flowers				
		Pollination Date 3/10/2012		Pollination Date 9/10/2012		
		Nova	Nadorcott	Nova	Nadorcott	Open pollinated
Female	**Marisol**	47	46	18	37	21
	Nules	43	43	19	19	20
	Nova	11	49	9	29	9

Mean number of seed per fruit

- Pollen parent and pollination time influence seed number and need to be considered when drawing conclusions about female fertility

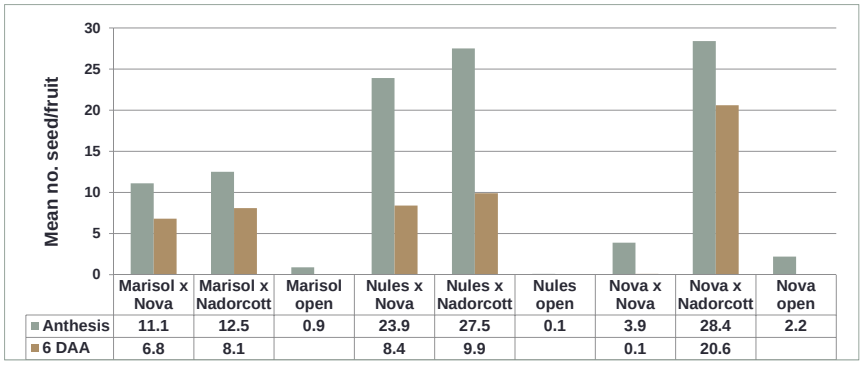
Pollination at anthesis (3 Oct 2012)								
	Mean no of seed/fruit				Mean no of seed/fruit			
	Nova				Nadorcott			
Female	Full	Small/ triploid	Abortive	**Total**	Full	Small/ triploid	Abortive	**Total**
Marisol	8.1	0.8	2.2	**11.1**	9.2	1.3	2.1	**12.5**
Nules	22.4	0.8	0.7	**23.9**	25.4	1.1	0.9	**27.5**
Nova	1.3	1.4	1.3	**3.9**	18.7	3.8	6.0	**28.4**

Pollination 6 days after anthesis (9 Oct 2012)								
	Mean no of seed/fruit				Mean no of seed/fruit			
	Nova				Nadorcott			
Female	Full	Small/ triploid	Abortive	**Total**	Full	Small/ triploid	Abortive	**Total**
Marisol	5.2	0.6	1.1	**6.8**	6.2	0.6	1.4	**8.1**
Nules	7.7	0.3	0.3	**8.4**	9.0	0.5	0.4	**9.9**
Nova	0.0	0.0	0.1	**0.1**	13.6	3.1	3.8	**20.6**



Total mean no. seed/fruit (full+small+abortive)

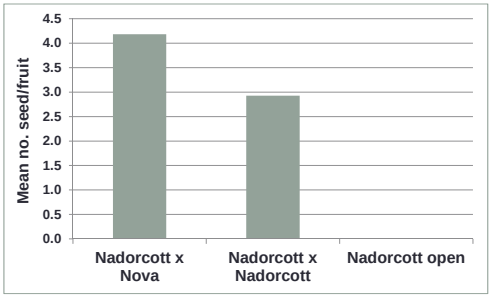
- Seed number varied with cultivars (male and female parent)
- Seed number reduced with later pollination date
- Open pollination showed lower seed number than controlled pollination



	Marisol x Nova	Marisol x Nadorcott	Marisol open	Nules x Nova	Nules x Nadorcott	Nules open	Nova x Nova	Nova x Nadorcott	Nova open
Anthesis	11.1	12.5	0.9	23.9	27.5	0.1	3.9	28.4	2.2
6 DAA	6.8	8.1		8.4	9.9		0.1	20.6	

2012 Nadorcott results from different trial (pollination at anthesis only)

- Controlled cross pollination caused higher seed number compared to self and open pollination



Summary

- Number of seed was higher in controlled manual cross pollinated flowers compared to open pollinated
- Number of seed reduced with later pollination date probably due to reduced stigma receptivity
- Fruit set potential was increased with controlled manual cross pollination
- Nadorcott as male parent caused higher number of seed in female parents compared to Nova as male parent
- Environmental factors were not taken into account in this study
- True seedlessness and female sterility can only be concluded if most environmental factors were eliminated during the test

	Seed formation	
	Yes	**No**
Open pollination	• Female fertile • Male fertile	• Possibly female sterile • Possibly male sterile
Self-pollination	• Female fertile • Male fertile	• Self-incompatibility • Male sterile
Controlled cross pollination (with standard testers)	• Female fertile	• Highly possible female sterile (seedless under normal conditions; low seeded (0-1 seed) under high pollen pressure)

Preliminary conclusion

- Seed formation is affected by genetic and environmental factors
- Open pollination is too prone to environmental factors and being first criteria for cultivar selection is insufficient as test method alone
- Female fertility is directly related to seediness
- Degree of female fertility can be estimated with higher accuracy/probability by controlled manual cross pollination
- However, to test strong female sterility, test methodology should be complemented by in vivo pollen germination studies and morphological studies on embryo sac degeneration to exclude incompatibilities
- In vivo pollen germination test does not necessarily allow assumptions on female fertility (reason for incompatibilities can be various)
- Comparison of percentage fruit set of self and controlled manual cross pollinated flowers can also allow assumptions on female fertility (however self-incompatibility level should be known/studied first)

- **Controlled manual cross pollinations are a good way to determine female fertility if done statistically to exclude environmental factors**
- **Clear definition of female fertility should be laid down**
- **Morphological studies of ovule and embryo sac necessary if controlled manual cross pollination results are inconclusive**

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THANK YOU!