Scientia Agricola
http://dx.doi.org/10.1590/1678-992X-2016-0129

'URS Campestre' seedless orange: a new mutant with female sterility

Renar João Bender[1]*, Rinaldo Pires dos Santos[2], Divanilde Guerra[3], Sergio Francisco Schwarz[1], Stefan da Silveira Bender[4]

[1]Federal University of Rio Grande do Sul – Horticultural Sciences Dept., Av. Bento Gonçalves, 7712 – 91540-000 – Porto Alegre, RS – Brazil.
[2]Federal University of Rio Grande do Sul – Biosciences Institute – Dept. of Botany. Av. Bento Gonçalves, 9500 - 91501-970 – Porto Alegre, RS – Brazil.
[3]Rio Grande do Sul State University – Campus Três Passos, R. Cipriano Barata 47, Três Passos – 98600-000, RS – Brazil.
[4]Federal University of Rio Grande do Sul/Faculty of Agronomy.
*Corresponding author <rjbe@ufrgs.br>

Edited by: Roberto Fritsche Neto

Received March 24, 2016
Accepted September 13, 2016

ABSTRACT: Seedlessness in fruit is a trait that is much sought after by juice making industries. Close to the city of São Sebastião do Caí, in the state of Rio Grande do Sul (RS), Brazil, a new mutant orange originating from natural mutation was identified and selected as a seedless material. To determine the mechanisms involved in the absence of seeds, the reproductive structures of this new mutant by comparison with a Valencia sweet orange as control, a cultivar with a profusion of seeds, was analyzed in terms of meiotic behavior, meiotic index, pollen viability, *in vitro* germination, and ovule features to determine the grounds for seed absence. Other morphological analyzes allowed for visualizing the structures of normal appearance and size in both cultivars. Meiotic analysis identified chromosome normal pairing with a predominance of bivalents at diakinesis and metaphase 1. URS Campestre flowers at different developmental stages had anthers and ovaries whose dimensions are typical while pollen grain analysis pointed to a standard developmental pattern, normal meiosis, high viability (84 %) and elevated *in vitro* pollen tube germination rates (63 %). The cv. Valencia and URS Campestre ovules had a similar shape and morphology, sharing an anatropous orientation, and two integuments. In the internal ovule analyses of Valencia sweet oranges, normal embryo sac cells were identified: presence of one egg cell and two synergids, three antipodes and a bigger and central cell containing two polar nuclei. However, the analysis of ovules from URS Campestre reveals an apparent senescence or non-formation of an embryo sac, where only a few highly stained and collapsed cells could be identified. These results led to the conclusion that female sterility in URS Campestre, with a total absence of a female gametophyte, is the limiting factor for fertilization and seed production.

Keywords: cytogenetic analysis, embryo sac abortion, female sterility, meiotic analysis, pollen viability

Introduction

A plant is seedless when it is not able to produce fruit with seeds, or produce the remains of aborted seeds or even fruit with very few seeds. Even fruits with no viable seeds should be considered seedless (Varoquaux et al., 2000). Absence of seeds in citrus fruit is a pleasing trait to fresh fruit consumers and a needed attribute for the juice industry (Hu et al., 2007; Ye at al., 2009). Some cultivars were selected and are of great value because of their seedlessness. Satsumas (*Citrus unshiu* Marc.), Clementines (*Citrus clementina* Hort. ex Tan.), Washington Navels (*Citrus sinensis* Osb.) and Tahiti lime (*Citrus latifolia* Tanaka) are leading cultivars in this segment (Breto et al., 2001; Deng et al., 1996; Zhang et al., 2012).

Most of these cultivars were obtained by way of selection of mutating buds or plantlets. Mutations set off changes in the genetic constitution at the ploidy level, in the chromosome structure and gene mutation generating seedless fruit (Deng et al., 1996). Despite the availability of seedless cultivars, one of the key targets of citrus breeding programs consists of hitting upon new cultivars with distinguishing attributes in addition to seedlessness to supply growing consumer demands (Wu and Zhao, 2004; Zhang et al., 2012).

In the usual course of events, seed development is a prerequisite to avoiding premature fruit drop and sustained subsequent fruit growth. Nonetheless, in a number of fruit species included in the *Citrus* genus, seedless fruit production (parthenocarpy) in the absence of fertilization, or as a result of embryo abortion, could occur (Koltunow, 1993; Vardi et al., 2008; Ye et al., 2009). In citrus species, different factors are associated with parthenocarpy such as polyploidy (Chen et al., 2004), male sterility (Hu et al., 2007; Yamamoto and Tominaga, 2002), self-incompatibility (Yamamoto and Tominaga, 2002; Ye et al., 2009; Zhang et al., 2012), abortion of ovule or embryo sac (Xiao et al., 2007; Zhou et al., 2011), adverse climatic factors including extreme, and both low and high temperatures (Hedhly, 2011; Thakur et al., 2010) in addition to hormone application (Talon et al., 1992) and neutron irradiation, 'x' or gamma rays (Bermejo et al., 2011; Vardi et al., 2008) , all of which could promote seedless fruit production.

In southern Brazil, a new seedless mutant orange, named 'URS Campestre' (*Citrus sinensis* Osbeck. URS Campestre) was identified and selected because of its seedless fruit (Figure 1). Acknowledging the significance of seedless material, the present work aimed to analyse the characteristics of the fruit and its reproductive structures to determine the embryological elements involved in the seedlessness of this new mutant.

Materials and Methods

Plant material

Flower buds at several developmental stages and

Figure 1 – Mature fruits of *Citrus sinensis* URS Campestre mutant. In the bottom row, in longitudinal (left) and transversal (right) cross sections. (São Sebastião do Caí, RS, Brazil).

pre-anthesis were collected in the first half of Sept (the normal flowering period in the Vale do Caí region) from six plants of the new mutant (URS Campestre) and from several trees of the control cultivar (*Citrus sinensis* Osbeck, cv. Valencia) at a grove located in São Sebastião do Caí, RS, the southernmost state in Brazil.

Along three fruiting seasons for about four months (starting at the end of June up to mid-Oct), every two or three weeks fruit samples were collected for quality analyses and determination of seed presence.

Cytogenetic and pollen grain analyses

Anthers from 25 pre-anthesis flowers of the URS Campestre mutant and cv. Valencia sweet oranges were extracted and visualized under a stereoscopic microscope concerning morphology and size.

For cytogenetic and pollen analyses, flower buds and pre-anthesis flowers were treated with Farmer's fixative (anhydrous ethanol/glacial acetic acid; 3:1, v/v) for 24 h and stored in 70 % (v/v) ethanol at -18 °C. Slides were prepared by squashing and staining all isolated anthers of a given flower bud in 2 % (w/v) propionic-carmine (Einhardt et al., 2006).

Meiotic analysis was performed on 25 flower buds from URS Campestre as well as from the control cultivar. All available pollen mother cells at any meiotic phase were analyzed. Cells with only bivalents (diakinesis and metaphase I) and regular disjunction (telophases and anaphases I and II) were considered normal. Cells with univalents, trivalents, quadrivalents or other associations (diakinesis and metaphase I) or with bridges, laggards and unequal disjunction (telophases and anaphases I and II) were recorded as abnormal.

The meiotic index (percentage of normal microspore tetrads) was determined using 25 flower buds and 100 microspore tetrads per flower of ´URS Campestre´ and of the control cultivar (Valencia). Tetrads with four equal-sized cells were considered as normal and any variant was labeled as abnormal.

Pollen viability was determined from 25 pre-anthesis flower buds and 100 mature pollen grains per flower in both the new mutant and the control cultivar. Well-stained pollen grains were considered viable and those unstained or feebly stained or empty were assigned as unviable.

Pollen *in vitro* germination was analyzed using Sahar and Spiegel-Roy (1984) culture medium (1 % of agar, 15 % sucrose, 100 ppm H_3BO_3, 1000 ppm $Ca(NO_3).4H_2O$, 300 ppm $MgSO_4.7H_2O$ and 100 ppm KNO_3). Freshly collected pollen grains were distributed on slides with culture medium and kept in germination chambers at 25 °C (± 2 °C) for 24 hours. Germination was analyzed in 1,000 grains from both Valencia and URS Campestre. Pollen grains with a pollen tube bigger than the grain diameter were considered germinated.

The analyses were performed under a bright field light microscope and images recorded by photomicrographs or digital image capturing.

Fruit quality

At every sampling period, 15 fruits of the URS Campestre mutant were picked and analyzed for fruit weight, peel color, titratable acidity, soluble solids, juice contents, peel thickness and number seeds.

Each fruit was individually weighed and peel color was determined with a handheld tristimulus chromameter (Konica/Minolta, model CR400) calibrated to white reference plate. The oranges were then cut in half and visually examined for seed presence and, afterwards, juice was extracted and analyzed for acidity and sugar content by titrating 6gr of juice with 0.1 M NaOH up to pH 8.2 and by a table refractometer, respectively.

Ovule analyses

Pre-anthesis flowers of the new mutant and of the control cultivar were fixed in 1 % glutaraldehyde and 4 % formaldehyde in 0.1 M phosphate buffer at pH 7.2 (McDowell and Trump, 1976). After ovary dissection, excised ovules were dehydrated in ethanol series, embedded in 2-hydroxyethyl-methacrylate acrylic resin (Gerrits and Smid, 1983), grouped and polymerized in Teflon molds (about 20 ovules per group). Three-micrometer thick sections were excised with a Leitz microtome equipped with a glass knife and mounted onto glass slides.

Longitudinal sections of the ovules were stained with a combination of periodic acid-Schiff (PAS) reaction and 0.05 % (w/v) Toluidine Blue O, pH 4.4 (O'Brien and McCully, 1981). Ovules (80) from the new seedless mutant URS Campestre and 60 ovules from the control cultivar (Valencia) were evaluated. The slides were observed under a bright field light microscope equipped with a digital camera.

Statistical analysis

Results of meiotic behavior, meiotic index, pollen viability, pollen *in vitro* germination and ovule analyses were compared by Tukey test at $p < 0.05$ using SAS (Statistical Analysis System, SAS Institute) version 6.4.

Results

Floral morphology

The flowers of URS Campestre and Valencia are perfect, with stamens and ovaries with similarity in size and morphology. After floral dissection, anthers with reduced sizes or even absent were not observed. In the ovary, no visual differences were observed in the number of ovules, insertion, size or total absence. Thus, in the new mutant, macro morphological characters showing signs of sterility in the androecium and gynoecium could not be identified.

Meiosis of microsporocyte

The analyses of meiotic behavior of both cultivars indicate a high percentage of normal cells in bivalent associations at diakinesis and metaphase I, besides typical disjunction at anaphase and telophase I and II (Figure 2A-C). There was a low frequency of irregularities with a presence of univalents, chromossome bridges and withheld chromossomes. The percentage of normal meiotic cells in URS Campestre and in Valencia was similar (Table 1).

Meiotic index analyses pointed towards high percentages of tetrads of microspores of similar size in both cultivars (Figure 2D) yielding a meiotic index of 79 % of normal cells in URS Campestre and 80 % in Valencia (Table 1). Comparing both cultivars, no significant differences were determined.

Pollen viability and germination percentage

The pollen grain analyses resulted in elevated percentages of normal pollen grains. Viable pollen grains were red-colored and filled with cytoplasm (Figure 2E-F). Viability percentages in both cultivars were similar and not significantly different: 84 % of pollen grains of URS Campestre were considered viable (Figure 2E) whereas the percentage for the pollen grains of cv Valencia was 85% (Figure 2F, Table 2).

In vitro pollen germination capacity for both the new mutant and the control cultivar was not statistically different (Table 3, Figures 2G and 2H).

Ovule and embryo sac structure

In the analyses of 80 ovules of cv. Valencia sweet oranges, no abnormalities were identified. The ovules are anatropous, crassinucellate and bitegmic. The nucellus contains cells that accumulate large amounts of starch grains in the micropylar zone (Figure 3A). Normal structure of the embryo sac cells with the presence of the egg apparatus (egg cell and two synergids), central cell and three antipodes were identified in all samples analyzed from this cultivar (Figure 3B).

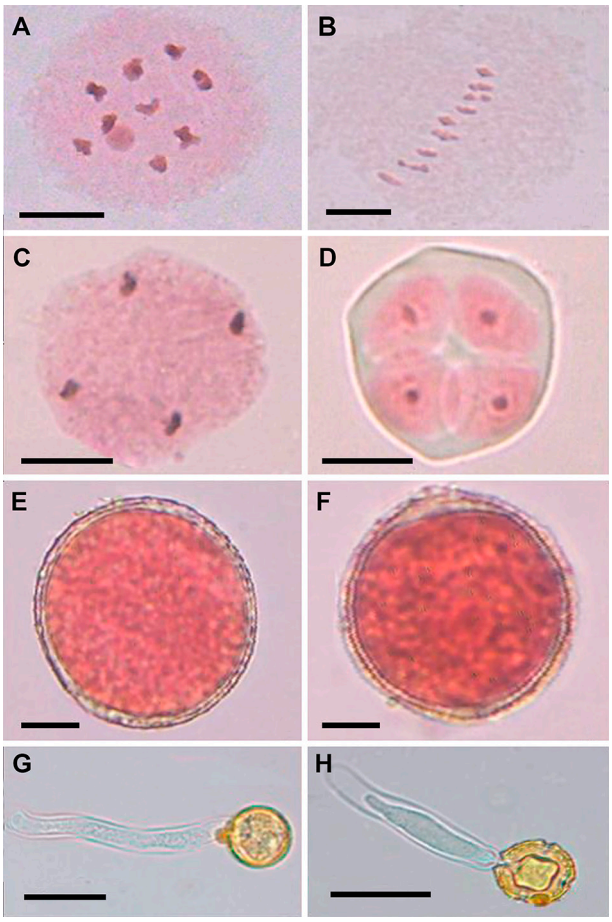

Figure 2 – A–D. Meiosis in microsporocytes from *Citrus sinensis* URS Campestre mutant. A. Microsporocyte at the prophase. B. Microsporocyte at the metaphase. C. Microsporocyte at the telophase II. D. Microspore tetrads with callose wall. E-F. Propionic-carmine pollen viability tests. E. *Citrus sinensis* URS Campestre mutant. F. *Citrus sinensis* cv. Valencia. G-H. Pollen tube growth viability. G. *Citrus sinensis* URS Campestre mutant. H. *Citrus sinensis* cv. Valencia. Scale bars: 10 µm (A-F); 50 µm (G-H).

Table 1 – Meiotic analysis and meiotic index for mutant oranges from URS Campestre and Valencia sweet oranges.

	Nº of meiotic cells	Nº of normal meiotic cells	% of normal meiotic cells	Nº of tetrads	Nº of normal Tetrads	Meiotic index
						%
URS Campestre	294	241	81.9 a	2558	2026	79.2 a
Valencia	262	216	82.4 a	2514	2019	80.3 a

Means within a column followed by the same letters are not significantly different at Tukey ($p > 0.05$).

Evaluations of 60 ovules of the URS Campestre mutant indicate typical morphological characters. Although the ovule morphology is the same as that found in cv. Valencia (crassinucellate and anatropous ovules with two integuments), abnormalities such as small changes in the nucellar length, slightly shorter than that of Valencia, and in the typical female gametophyte were visualized. Thus, despite the similar starch accumulation in the nucellus (Figure 3C), an abnormal development occurs inside the megasporangium (nucellus), with a total absence of typical embryo sac cells, evidenced by the intense staining reaction of cell remnants, and the impossibility to identify embryo sac nucleus (Figure 3D). This abnormal feature, with minor variations (number of, and extension of collapsed cells), was found in all URS Campestre ovules analyzed.

Fruit quality

Petry et al. (2012) recommend harvesting sweet oranges under southern Brazilian conditions after reaching sugar to acid ratios of at least eight. Based on this value URS Campestre should be harvested under the same settings in the second half of Aug. In that period, the fruit quality from URS Campestre was similar to that of Valencia sweet oranges harvested at the same time (Table 4).

Table 2 – Comparison of pollen grain viability between URS Campestre and Valencia sweet oranges.

	Nº of Pollen grains	Nº of viable Pollen grains	% of viable pollen grains
URS Campestre	2506	2110	84.2 a
Valencia	2523	2137	84.7 a

Averages within a column followed by the same letters were not significantly different at Tukey ($p > 0.05$).

Table 3 – Comparison of germinating frequency of pollen grains between URS Campestre and Valencia sweet oranges.

	Nº of pollen grains	Nº of germinated pollen grains	Pollen germination
			%
URS Campestre	1008	638	63.3 a
Valencia	1000	628	62.8 a

Averages within a column followed by the same letters were not significantly different at Tukey ($p > 0.05$).

Table 4 – Quality parameters of mutant oranges from URS Campestre harvested over three seasons: In 2010, oranges were picked on Aug 26, in 2011 on Aug 13, and in 2012 on Aug 03.

Year	Average fruit weight	Peel thickness	Juice contents	Soluble solids	Acidity
	g	mm	%	°Brix	% citric acid
2010	197.2	3.32	46.62	11.13	1.41
2011	167.58	4.23	51.94	10.18	1.18
2012	212.21	4.57	52.99	12.5	1.31
Cv. Valencia*	199.54		50.05	9.15	1.02

*Source: Petry et al. (2012).

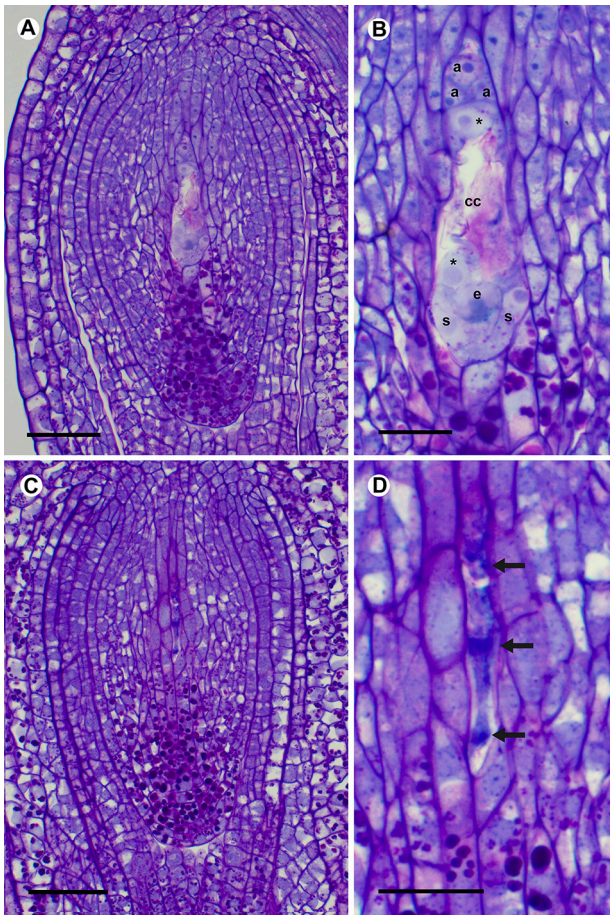

Figure 3 – A. Longitudinal section of the mature ovule of *Citrus sinensis* cv. Valencia. B. Detail of A. This normal embryo sac contains two synergids (s), the egg cell (e), three antipodal cells (a), and a large central cell (cc) containing two polar nuclei (asterisks). C. Longitudinal section of the mature ovule of *Citrus sinensis* URS Campestre mutant. In both, the micropylar pole of the nucellus has a similar amount of starch grains (periodic acid-Schiff reaction and Toluidine Blue staining). D. Detail of C. This abnormal development shows only collapsed cells with intensely stained cytoplasm (arrows). Scale bars: 50 µm (A and C); 20 µm (B and D).

As for seed presence, in the years 2011 and 2012 only one orange in each year presented just one seed. In the year 2010, no seeds were observed in any fruit after seven sampling times.

In the following two years of evaluations, six samples were picked every year. The sampled oranges were also evaluated with regard to its roundness. In the three years of evaluation, sphericity values ranged from 0.98 to 1.01.

Peel color of URS Campestre does not differ from the color of other sweet orange cultivars. The citrus color index calculated for URS Campestre as indicated by Jimenez-Cuesta et al. (1981) ranged from 4.34 up to 4.84 at the indicated harvest date. Chroma values for the

same sampling period ranged from 72.11 up to 78.52, *Hue* angle values from 72.16 to 74.65 and luminosity, as indicated by L* values from the chromameter CR400, from 63.20 to 66.59.

Discussion

Securing possession of seedless citrus cultivars is important for citrus breeding programs for the purpose of either offering unique cultivars to the market or handing over this source of variability in support of novel crossings to breeding programs. In view of the uniqueness of URS Campestre, the mechanisms involved in its seedlessness were investigated.

In citrus species, bud and plantlets mutations are fairly widespread. At the point in time when these mutations become stable, they may give rise to new selections with distinct characteristics (Asíns et al., 1999; Breto et al., 2001; Ye et al., 2009; Zhang et al., 2012). This description corresponds to what was observed in the case of URS Campestre and this new selection in all probability is a result of a somatic mutation. Because of the distinguishing characteristics of its fruit size, color and seedlessness it was selected for this study (Figure 1).

Similar to the present study, mutations contributing to the development of cultivars without seeds have already been described in the literature. Hu et al. (2007) and Ye et al. (2009) refer to mutations in tangerines while Zhang et al. (2012) describe mutations in lemons. In citrus species, which underwent mutations, fruit production in the absence of seeds is only possible through parthenocarpy (Koltunow, 1993; Vardi et al., 2008).

According to Talon et al. (1992) and Gorguet et al. (2005), deviations in hormonal equilibrium might as well promote parthenocarpy. Furthermore, Ortiz (2002) refers to specific hormones in ovary tissues, which determine fruit development in the absence of fertilization. Many factors might contribute to anomalous development or abortion of seeds in citrus species. Vardi et al. (2005) stated that somatic mutations at the ploidy level might result in nonstandard chromossome paring at meiosis and trigger pollen sterility. Moreover, according to Chen et al. (2004), chromosome irregularities could reduce or hamper pollen production and disturb the pollination processes, a phenomenon observed by Hu et al. (2007) in a seedless tangerine cultivar. Lin et al. (1995) and Hu et al. (2007) have described male sterility as being responsible for the absence of seeds in citrus cultivars. Climatic conditions, such as temperature and water deficit, might also influence reproductive factors and for this reason have an effect on seed production.

Vardi et al. (2008) concluded that a citrus cultivar is regarded as seedless when it shows evidence of an aptitude to yield standard and typical fruit without seeds or with aborted seeds or an extremely reduced number of seeds. Over three years of evaluations URS Campestre did not produce seeds. For this reason that new mutant should be labeled as a seedless cultivar.

Thakur et al. (2010) and Hedhly (2011) indicated that ambiental stresses on plants that are in the reproductive stage might have an effect on meiotic processes and impede viable pollen production. Moreover, according to Boyer and McLaughlin (2007) initial stages of cells at meiosis are more responsive to stresses caused by adversity. Unfavorable factors damage tapetal cells compromising nutrition and the development of meiocytes, resulting in smaller anthers, and with hypertrophy cause abortion of pollen grains obstructing pollination. According to Nakano et al. (2001) abnormalities in the meiotic processes cause unviability of pollen grains and degenerating anthers resulting in plant sterility.

In the present study, the hypothesis of changes at ploidy level and meiotic abnormalities were evaluated through counts of chromosome numbers per cell and the evaluation of their behavior at meiosis I and II. Abnormalities of meiotic behavior and pollen grain sterility were described by Lin et al. (1995) and Hu et al. (2007) as being the main factors responsible for the absence of seeds in citrus fruit. The observations indicate that all cells are diploid ($2n = 18$) with 18 chromosomes and, in most cases, show evidence of typical meiotic performance (Figure 2). High percentages of standard meiotic cells were observed in URS Campestre and Valencia (Table 1) and, additionally, viable pollen grains with adequate germination rates (Figure 2, Tables 2 and 3) for the autopollination course.

With the results of the analyses of these factors (Figure 2, Tables 1 up to 4) the hypothesis that changes in the level of ploidy, abnormal chromossome paring at meiosis and pollen grain sterility might be the cause of the absence of seeds in URS Campestre should be disregarded. Likewise, the hypothesis of sterility as a consequence of ambient factors should also be rejected.

Pollen grain viability might also be affected by hormone sprays such as auxins and gibberellins (Tallon et al., 1992). Yet, these hormones have not been applied to plants and, for this reason, should not have had an effect on pollen grain viability (Table 2).

An additional characteristic that could hinder seed production in citrus fruit is auto incompatibility. Auto-incompatibility derives from the failure in fecundation processes by way of pollen from the same plant or with pollen from plants with the identical genetic constitution of the receptor plant (Chai et al., 2011; Distefano et al., 2009; Miao et al., 2011; Ye et al., 2009). The grove at which URS Campestre plants are located has many other citrus cultivars (cv. Valencia and Piralima sweet oranges; cv. Ponkan mandarin, Murcott tangor and Montenegrina willowleaf mandarin and Okitsu satsumas) surrounding the trees from which the flowers and fruit were collected. Even with plenty of pollen available, URS Campestre fruit were seedless.

Abnormalities in the development of the embryo sac and, as a consequence, its sterility might be associated with the absence of seeds in citrus species (Frost and Soost, 1968). In satsumas and navel oranges, fe-

male gametophytic sterility derives from failure of embryo sac development either because of senescence of the megaspore mother cells or because of embryo sac formation in the early stages of meiotic division, *i. e.*, before the formation of megaspores which inhibit the production of seeds in fruit (Hodgson, 1967). Xiao et al. (2007) and Zhou et al. (2011) identified degeneration in tangerines of the embryo sac mother cells (viable megaspores) leading to their sterility and subsequent seedlessness.

Moreover, gametic female sterility may be partial or total. In Tahiti lime (*C. latifolia* Tanaka), Shamouti sweet orange (*C. sinensis* (L.) Osbeck.) and grapefruit (*C. paradisi* Burm.), in spite of either the absence of a large majority of embryo sacs or abortive ovules, a reduced number of embryo sacs might mature and become functional and occasionally yield seed formation (Frost and Soost, 1968). Wilms et al. (1983) analyzed aspects of female sterility in *Citrus limon*. This species has both fertile and sterile flowers, which have externally atrophied pistils with many internal cellular alterations, and poorly developed ovules. In fertile flowers, most ovules show aberrant development, with degeneration of either the megaspore mother cells or the viable megaspore. In some cases, embryo sacs manage to develop completely, but degenerate before fertilization.

In the present study, analyses of ovules of cv. Valencia flowers were representative of typical embryo sac cells. On the other hand, the analyses of URS Campestre flowers point towards abnormal development or senescence of embryo sacs characterized by the absence of typical cells for fecundation processes and embryo development. Rather, the presence of cell remains of the seven embryo sac cells shows that there was a disruption of the normal process of megasporogenesis or megagametogenesis. The similarity in nucellar size in both cultivars (shorter in URS Campestre) could suggest that the problem occurs during megagametogenesis, when we should expect a much shorter nucellus if a developmental error happened during megasporogenesis. However, the formation of the nucellus, and the ovule as a whole (including the integuments), do not depend on megasporogenesis and megagametogenesis occuring, as was concluded by Frost and Soost (1968) and Wilms et al. (1983).

Conclusion

In the present study, URS Campestre presented typical meiosis in addition to high pollen grain viability and germination to suffice for pollination processes. Cytological analyses carried out with microsporangia of the new mutant identified normal meiotic cells as well as elevated *in vitro* germination of pollen grains that could support polinization and fertilization processes. Consequently, the idea of male sterility as being the reason for seedlessness in URS Campestre should be outrightly rejected.

The new mutant orange has well-developed flowers, with normal anthers, ovary, and ovules morphologically similar to the control cultivar (Valencia sweet orange). Thus, the grounds for the nonexistence of seeds is associated with abnormalities in the formation of the female gametophyte (embryo sac) leading to a complete failure in the fertilization process. In fact, the URS Campestre orange is a new mutant with female sterility.

The cytological characteristics of the nucellar contents in the new mutant URS Campestre do not allow for determining if the cell debris were abortive megaspore mother cells, abortive megaspores or complete degenerative embryo sacs. Therefore, only a complete embryological evaluation of floral buds at different stages of development would allow for the precise identification of which step (sporogenesis or gametogenesis) is stopping the embryo sac formation.

Despite seedlessness, the fruit quality of URS Campestre is comparable to Valencia sweet orange, the leading cultivar growing in the same region of the state of Rio Grande do Sul, Brazil.

References

Asíns, M.J.; Monforte, A.J.; Mestre, P.F.; Carbonell, E.A. 1999. Citrus and Prunus copia-like retrotransposons. Theoretical and Applied Genetics 99: 503-510.

Bermejo, A.; Pardo, J.; Cano, A. 2011. Influence of gamma irradiation on seedless Citrus production: pollen germination and fruit quality. Food and Nutrition Sciences 2: 169-180.

Boyer, J.S.; McLaughlin, J.E. 2007. Functional reversion to identify controlling genes in multigenic responses: analysis of floral abortion. Journal of Experimental Botany 58: 267-277.

Breto, M.P.; Ruiz, C.; Pina, J.A.; Asíns, M.J. 2001. The diversification of *Citrus clementina* Hort. ex Tan., a vegetatively propagated crop species. Molecular Phylogenetics and Evolution 21: 285-293.

Chai, L.J.; Ge, X.X.; Biswas, M.K.; Xu, Q.; Deng, X.X. 2011. Self-sterility in the mutant 'Zigui shatian' pummelo (*Citrus grandis* Osbeck) is due to abnormal post-zygotic embryo development and not self-incompatibility. Plant Cell, Tissue and Organ Culture 104: 1-11.

Chen, C.L.; Guo, W.W.; Yi, H.L.; Deng, X.X. 2004. Cytogenetic analysis of two interspecific Citrus allotetraploid somatic hybrids and their diploid fusion parents. Plant Breeding 123: 332-337.

Deng, X.X.; Guo, W.W.; Sun, X.H. 1996. Advances in breeding and selection of seedless types of citrus in China. Acta Horticulturae Sinica 23: 235-240.

Distefano, G.; Caruso, M.; Malfa, S.L.; Gentile, A.; Tribulato, E. 2009. Histological and molecular analysis of pollen-pistil interaction in clementine. Plant Cell Reports 28: 1439-1451.

Einhardt, P.M.; Correa, E.R.; Raseira, M.C.B. 2006. Comparison among methods for testing pollen viability. Revista Brasileira de Fruticultura 28: 5-7 (in Portuguese, with abstract in English).

Frost, H.B.; Soost, R.K. 1968. Seed reproduction: development of gametes and embryos. p. 290-324. In: Reuther, W.; Batchelor, L.D.; Webber, H.J., eds. The citrus industry. University of California Press, Berkeley, CA, USA.

Gerrits, P.O.; Smid, L. 1983. A new, less toxic polymerization system for the embedding of soft tissues in glycol methacrylate and subsequent preparing of serial sections. Journal of Microscopy 132: 81-85.

Gorguet, B.; van-Heusden, A.W.; Lindhout, P. 2005. Parthenocarpic fruit development in tomato. Plant Biology 7: 131-139.

Hedhly, A. 2011. Sensitivity of flowering plant gametophytes to temperature fluctuations. Environmental and Experimental Botany 74: 9-16.

Hodgson, R.W. 1967. Horticultural varieties of citrus. p. 31-59. In: Reuther, W.; Webber, H.J.; Batchelor, L.D., eds. The citrus industry. University of California Press, Berkeley, CA, USA.

Hu, Z.Y.; Zhang, M.; Wen, Q.G.; Wei, J.; Yi, H.L.; Deng, X.X.; Xu, X.H. 2007. Abnormal microspore development leads to pollen abortion in a seedless mutant of 'Ougan' mandarin (*Citrus suavissima* Hort. Ex Tanaka). Journal of the American Society for Horticultural Science 132: 777-782.

Jimenez-Cuesta, M.; Cuquerella, J.; Martinez-Javega, J.M. 1981. Determination of a color index for citrus fruit degreening. Proceedings of the International Society for Citriculture 2: 750-753.

Koltunow, A.M. 1993. Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. The Plant Cell 5: 1425-1437.

Lin, H.Y.; Zheng, J.Q.; Chen, G.X.; Lai, Z.X. 1995. Advances in the sterility of citrus. Fujian Fruits 4: 13-15.

McDowell, E.M.; Trump, B. 1976. Histological fixatives for diagnostic light and electron microscopy. Archives of Pathology & Laboratory Medicine 100: 405-414.

Miao, H.X.; Qin, Y.H.; Silva, J.A.T.; Ye, Z.X.; Hu, G.B. 2011. Cloning and expression analysis of S-RNase homologous gene in *Citrus reticulate* Blanco cv. Wuzishatangju. Plant Science 180: 358-367.

Nakano, M.; Nesumi, H.; Yoshioka, T.; Yoshida, T. 2001. Segregation of plant with undeveloped anthers among hybrids derived from the seed parent, 'Kiyomi' (*Citrus unshiu* x *C. sinensis*). Journal of the Japanese Society for Horticultural Science 70: 539-545.

O'Brien, T.P.; McCully, M.E. 1981. The Study of Plant Structure. Principles and Selected Methods. Termacarphi Pty, Melbourne, Australia.

Ortiz, J.M. 2002. Botany: taxonomy, morphology and physiology of fruits, leaves and flowers. p. 16-35. In: Dugo, G., Giacomo, A., eds. Citrus: the genus Citrus. CRC Press, Boca Raton, FL, USA.

Petry, H.B.; Koller, O.C.; Bender, R.J.; Schwarz, S.F. 2012. Fruit quality of ´Valencia´ oranges harvested from organic and conventional production systems. Revista Brasileira de Fruticultura 34: 167-174 (in Portuguese, with abstract in English).

Sahar, N.; Spiegel-Roy, P. 1984. *In vitro* germination of avocado pollen. Scientia Horticulturae 19: 886-888.

Talon, M.; Zacarias, L.; Primo-Millo, E. 1992. Gibberellins and parthenocarpic ability in developing ovaries of seedless mandarins. Plant Physiology 99: 1575-1581.

Thakur, P.; Kumar, S.; Malik, J.A.; Berger, J.D.; Nayyar, H. 2010. Cold stress effects on reproductive development in grain crops: an overview. Environmental and Experimental Botany 67: 429-443.

Varoquaux, F.; Blanvillain, R.; Delseny, M.; Gallois, P. 2000. Less is better: new approaches for seedless fruit production. Trends in Biotechnology 18: 233-242.

Vardi, A.; Levin, I.; Carmi, N. 2008. Induction of seedlessness in Citrus: from classical techniques to emerging biotechnological approaches. Journal of the American Society for Horticultural Science 133: 117-126.

Wilms, H.J; Van Went, J.L.; Cresti, M.; Ciampolini, F. 1983. Structural aspects of female sterility in *Citrus limon.* Acta Botanica Neerlandica 32: 87-96.

Wu, J.H.; Zhao, X.Y. 2004. The seedless mechanism of wampee and its main cultivars. Journal of Guangxi Agricultural and Biological Science 35: 116-117.

Xiao, J.P.; Tan, J.J.; Liu, H.L.; Chen, L.G.; Ye, W.Q.; Cheng, W.L. 2007. Studies on the seedless mechanism of Lipeng No. 2 Ponkan (*Citrus reticulata*). Journal of Fruit Science 24: 421-426.

Yamamoto, M.; Tominaga S. 2002. Relationship between seedlessness of keraji (*Citrus keraji* hort. ex Tanaka) and female sterility and self-incompatibility. Journal of the Japanese Society for Horticultural Science 71: 183-186.

Ye, E.; Qin, Y.; Ye, Z.; Silva, J.A.T.; Zhang, L.; Wua, X.; Lin, S.; Hua, G. 2009. Seedless mechanism of a new mandarin cultivar 'Wuzishatangju' (*Citrus reticulata* Blanco). Plant Science 177: 19-27.

Zhang, S.W.; Huang, G.X.; Ding, F.; He, X.H.; Pan, J.C. 2012. Mechanism of seedlessness in a new lemon cultivar 'Xiangshui' [*Citrus limon* (L.) Burm. F.]. Sexual Plant Reproduction 25: 337-345.

Zhou, X.; You, S.; Zhang, Y.; Bian, D.; Yang, X.; Zhang, Y. 2011. Embryological research on the ovule abortion development of super late-maturing cultivar JZT(P)-1 tangerine. Chinese Agricultural Science Bulletin 27: 231-234.