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ARTICLE

Reproductive characteristics of citrus rootstocks grown under greenhouse and field environments

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Abstract – *The aim of the present study was to evaluate the possible effect of environmental factors on meiosis, meiotic index, pollen viability and in vitro germination of pollen from stock plants of the rootstocks Trifoliate, 'Swingle', 'Troyer', 'Fepagro C13', 'Fepagro C37' and 'Fepagro C41' grown in a protected environment in comparison with stock plants grown in the field. The results showed that values for the characteristics analyzed in 2008, 2009 and 2010 were always higher in the field than in the greenhouse conditions. In the field, the average of normal meiotic cells was 60.05%, 44.44% and 60.12%, respectively, and in the greenhouse, 52.75%, 30.95% and 52.82%, respectively. Mean pollen viability in the field was 90.28%, 56.23% and 74.74%, and, in the greenhouse, 64.25%, 41.41% and 66.71%, respectively. As temperature oscillations were higher in the greenhouse than in the field, we suggest that this negatively affects the reproductive characteristics analyzed.*

Key words: *Citriculture, meiotic behavior, pollen fertility, pollen germination.*

INTRODUCTION

Among fruits for *in natura* consumption, citrus fruits are in first place in regard to production volume, and citriculture stands out worldwide as one of the most important agricultural and agroindustrial activities (Boteon and Neves 2005, FAO 2012). The fruits are also used for industrialization (Donadio et al. 2005). Higher citrus production as compared to other fruit crops is based on cultivated area and on the increasing use of productive scion and rootstock cultivars adapted to local environmental conditions and resistance to diseases and pests (Boteon and Neves 2005, Donadio et al. 2005, Hussain et al. 2011).

The choice of a rootstock is of extreme importance as it may affect several characteristics of the scion plant and fruits. Factors such as climate, soil, scion variety, management and good plant health conditions must be considered (Pompeu Junior 2005). In order to assure genetic and plant health conditions, it is better if rootstock plants are grown in protected environments (greenhouses) from buds or seeds from certified stock plants (Maciel et al. 2008). Stock plants from seeds do not necessarily need to be grown under protected conditions, but this practice assures healthier plants (Carvalho et al. 2000). In citrus, rootstock mother plants should have good production of viable seeds in order to ensure a large number of plants produced (Moreira et al. 2010). Even if reproduction is mainly by nucellar embryony, viable pollen is necessary for endosperm formation to ensure embryo nutrition and development (Koltunow 1993). Environmental factors in greenhouses may hinder reproductive characteristics and therefore seed and fruit production. According to Thakur et al. (2010) and Hedhly (2011), environmental stresses in plants during reproductive stages may be detrimental to the meiotic process and to viable pollen production. The initial stages of development of meiotic cells are most sensitive to stresses that also affect tapetum cells, therefore hindering cell nutrition and anther development, leading to pollen abortion and unviable pollination (Boyer and McLaughlin 2007).

The aim of the present study was to evaluate the possible effect of environmental factors on meiosis, meiotic index, pollen viability and *in vitro* germination of stock plants of citrus rootstocks grown in a protected environment in comparison with plants grown in the field.

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MATERIAL AND METHODS

The rootstocks Trifoliate (*Poncirus trifoliata* (L.) Raf,), Citrumeleiro 'Swingle' (*Citrus paradisi* Macf. x *P. trifoliata*) and Citrangeiro 'Troyer' (*C. sinensis* Osb. x *P. trifoliata*), widely used in citrus orchards in Brazil, as well as three new hybrids (*C. sinensis* x *P. trifoliata*), 'Fepagro C13' 'Fepagro C37' and 'Fepagro C41', which have potential in diversifying rootstocks in citrus orchards, were used in this study.

Flower buds in several stages of development were collected in the field at Fazenda Panoramas Citros, Butiá, Rio Grande do Sul, Brazil (lat 29° 57' S, long 51° 40' W), and under a protected environment at EEA-UFRGS (Estação Experimental Agronômica, Universidade Federal do Rio Grande do Sul), Eldorado do Sul, Rio Grande do Sul, Brazil (lat 30° 29' S, long 51° 06' W). The plants in the field were exposed to natural climatic conditions. The protected environment was a greenhouse with a metallic structure and plastic roof. The plants were grown in 100 L capacity pots with a substrate composed of soil, acacia bark residue and carbonized rice hull (2:2:1-v:v:v), with daily drip irrigation. 'Fepagro C41' (*C. sinensis* x *P. trifoliata*) was only studied under greenhouse conditions, as no plants of this rootstock were available in the experimental field.

Temperature was monitored throughout the flowering period by a meteorological station (EEA-UFRGS) in the field and by a thermohygrograph in the greenhouse.

For cytogenetic analyses, flower buds were collected in 2008, 2009 and 2010, fixed in ethanol:acetic acid (3:1) for 24 h and stored in 70% ethanol in a freezer. Slides were prepared by squashing and staining in 2% propionic carmine all the anthers of a given flower bud.

Meiotic analysis was performed on ten flower buds per rootstock per environment per year; the slides were prepared with all the anthers of each flower bud. Ten replications for each rootstock and location were evaluated. All available pollen mother cells of any meiotic phase were analyzed. Cells with only bivalents (diakinesis and metaphase I) and regular disjunction (telophases and anaphases I and II) were considered as normal. Those with univalents, trivalents, quadrivalents and other associations (diakinesis and metaphase I) or with bridges, laggards and unequal disjunction (telophases and anaphases I and II) were recorded as abnormal.

Meiotic index, percentage of normal pollen tetrads, was determined using ten flower buds from a total of ten replications per rootstock and location, and 1000 post-meiotic products per rootstock per environment per year. Tetrads with four equal-sized cells were considered as normal, and any variant as abnormal.

Pollen viability was determined in ten flower buds. Slides were prepared with all the anthers of each flower bud. Ten replications for each rootstock and location were evaluated. A total of 10,000 mature pollen grains per rootstock per location per year were evaluated. Well-stained pollen grains were considered viable (Figure 1i), and those unstained, weakly stained or empty as unviable (Figure 1r).

Pollen *in vitro* germination was analyzed in 2010, using the Sahar and Spiegel-Roy (1984) culture medium (1% agar, 15% sucrose, 100ppm H_3BO_3, 1000ppm $Ca(NO_3).4H_2O$, 300ppm $MgSO_4.7H_2O$ and 100ppm KNO_3). Four flower buds of each genotype under field and greenhouse conditions were evaluated, with four replications per rootstock and location. Freshly collected pollen grains were distributed in slides with culture medium and kept in a BOD (Biochemical Oxygen Demand) germination chamber at 25 ± **2 °C**, for 24 hours. Germination was analyzed in 1000 grains per rootstock per location. Pollen grains with a pollen tube bigger than pollen grain diameter were considered as germinated. In 40 germinated grains, pollen tube length (from the exine to the tube apex) was measured.

Meiotic behavior, meiotic index, pollen viability, pollen *in vitro* germination and pollen tube length analysis were performed directly under an optical microscope, and images were recorded by photomicrographs or digital image capturing. Results were analyzed by the Tukey test at 5% with the aid of SAS software.

RESULTS

Through analysis of meiotic cells, it was confirmed that all rootstocks were diploid (n=9, therefore 2n=18) (Figure 1a and b). All phases of meiosis I and II were recorded, both for regular and irregular behavior (Figure 1).

Variation in percentages of normal cells was observed among rootstocks grown under field and greenhouse conditions, as well as among years (Table 1). Percentages of normal cells were always higher in plants under field conditions than those under greenhouse conditions. Under field conditions, percentages of normal cells for Trifoliate were 62.77%, 48.48% and 61.78% in 2008, 2009 and 2010, respectively, and, under greenhouse conditions, 58.52%, 35.29% and 55.93% in 2008, 2009 and 2010, respectively. All other rootstocks performed in the same way (Table 1), with higher percentage of normal cells under field conditions than under greenhouse conditions. Considering years, values were similar for all rootstocks under field and greenhouse conditions for the years 2008 and 2010. In 2009, percentages of normal cells were lower than for 2008 and 2010 for all the rootstocks in the field and in the greenhouse (Table 1). The averages of normal cells for all rootstocks were 60.05% and 60.12% in 2008 and 2010, respectively, and 44.44% in

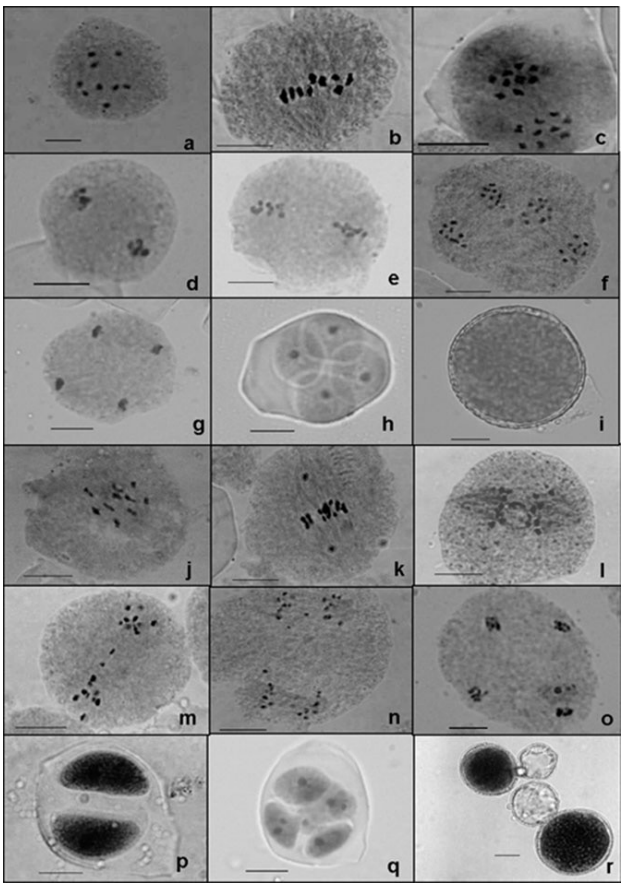

Figure 1. Normal and irregular cells from meiosis I to pollen. a) Diakinesis with 9 bivalents (II); b) Metaphase I with 9II; c) Anaphase I with normal disjunction (9/9); d) Telophase I; e) Metaphase II; f) Anaphase II with normal disjunction (9/9/9/9); g) Telophase II; h) tetrad; i) mature viable pollen grain; j) Diakinesis with 8 II and 2 I (univalents); k) Metaphase I with 6 II, 2 I and 1 IV (quadrivalent); l) Anaphase I with laggards; m) Telophase I with laggards; n) Anaphase II with irregular disjunction; o) Telophase II with micronuclei; p) Dyad; q) Polyad; r) Unviable (not stained) pollen grains. Scale bar =10μm.

2009 under field conditions. Under greenhouse conditions, the averages were 52.75% and 52.82% in 2008 and 2010, respectively, and 30.95% in 2009 (Table 1).

Besides normal pollen tetrads (Figure 1 h), dyads (Figure 1 p), polyads (Figure 1 q) and microcytes were observed during meiotic index analyses. Differences between plants under field and greenhouse conditions, as well as among years were also observed, with higher values being found under field conditions (Table 2). Under field conditions, the highest meiotic indexes were observed for Trifoliate: 64.24% in 2008, 52.35% in 2009 and 64.09% in 2010, while in the greenhouse the values were lower, 62.14% in 2008, 35.29% in 2009 and 62.63% in 2010. The same differences between field and greenhouse were observed for all the other genotypes (Table 2). Among years, under field conditions, the averages were 61.28% in 2008, 46.31% in 2009 and 61.28% in 2010, and, under greenhouse conditions, 54.26% in 2008, 31.75% in 2009 and 54.47% in 2010 (Table 2).

Trifoliate presented the highest percentages of viable pollen grains under both field and greenhouse conditions. In the field, the values were 95.12% in 2008, 63.47% in 2009 and 97.76% in 2010 and, in the greenhouse, 68.13%, 49.45% and 78.40% in 2008, 2009 and 2010, respectively (Table 3). Higher values for pollen fertility in the field were observed for all the other genotypes (Table 3). The averages for all rootstocks among years were 90.28% in 2008, 56.23% in 2009 and 74.74% in 2010 in the field, and 64.25% in 2008, 41.41% in 2009 and 66.71% in 2010 in the greenhouse (Table 3). Low frequencies of pollen grains bigger than normal ones were observed, but they were not considered as unreduced grains, as they were not 30 to 40% larger than the normal ones, a criterion normally used to classify unreduced gametes (Hermsen 1984). Therefore, for meiotic behavior, meiotic index and pollen fertility, plants under field conditions performed better that those under greenhouse conditions, and lower values for all genotypes in both conditions were observed in 2009.

Pollen *in vitro* germination and pollen tube length for all plants were higher under field than under greenhouse conditions. Averages for pollen *in vitro* germination for all genotypes were 41.81% in the field and 36.32% in the greenhouse and, for pollen tube length, 45.59 μm in the field and 21.78μm in the greenhouse (Table 3).

DISCUSSION

For all the characteristics studied, plants grown under field conditions performed better than those under greenhouse conditions (Tables 1, 2 and 3), leading to the conclusion that environmental factors in the protected environment negatively affect the reproductive characteristics analyzed. In the greenhouse, the minimum and maximum temperature values were higher and the oscillations more marked than in the field (Figure 2). According to Nayyar et al. (2005), Zinn et al. (2010) and Hedhly (2011), sudden variations in temperature in the reproductive stage, even for a short period, are enough to damage the cells and hinder meiosis and pollen fertility. In several plants, such as rice (*Oryza sativa* L.) (Oliver et al. 2005), peach (*Prunus persica* L) (Kozai et al. 2004, Nava et al. 2009), apricot (*Prunus armeniaca* L.) (Rodrigo and Herrero 2002), citrus (*Citrus unshiu*) (Takagi et al. 1982), cherry (*Prunus avium* L.) (Hedhly et al. 2007) and barley (*Hordeum vulgare* L.) (Sakata et al. 2000), unfavorable temperatures are reported to impair meiotic behavior and pollen fertility.

Cells with irregularities were already observed in meiosis I, corroborating Thakur et al. (2010) that environmental stresses are detrimental as of the first stages of meiosis.

The abnormalities found at meiosis II, pollen tetrads and reduced pollen viability is in agreement with Chen et al. (2004) and Kamiri et al. (2011) that, in citrus, abnormalities at meiosis I lead to abnormalities in meiosis II, pollen tetrads and, therefore, low pollen viability. Agarwal (1987) also suggested that in citrus there is a positive correlation between frequency of univalents and pollen sterility. Abnormalities in the meiotic process, leading to reduced pollen viability, has been described in other plants, such as *Zea mays*, Paspalum (*Paspalum* spp.), triticale (x *Triticosecale* Wittmack), soybean (*Glycine max*) and brassicas (*Brassica napus* and *B. Campestris*) (Bione et al. 1999, Pagliarini 2000, Guerra et al. 2011).

The higher percentage of meiotic irregularities and lower pollen fertility observed in the greenhouse (Tables 1, 2 and 3) may be a limiting factor in producing citrus rootstock mother plants from seeds in protected environments, due to the possible negative effect on seed production. Even considering that these plants reproduce by apomixis (Cameron and Soost 1969, Koltunow 1993), viable pollen grains are necessary for fertilization and endosperm development of the sexual embryo, therefore assuring the essential nutrients and hormones for nucellar embryo growth and viable seed formation (Koltunow 1993, Davies and Albrigo 1994, Spielman et al. 2003, Machado et al. 2005). Low percentage of viable pollen and, consequently, low seed production has been observed in orange, mandarin and lime by Moreira and Gurgel (1941) and Sellito-Boaventura and Pio (1989). The lower values for the characteristics analyzed in the present study in the greenhouse (Tables 1, 2 and 3) show that environmental factors in the greenhouse are negatively

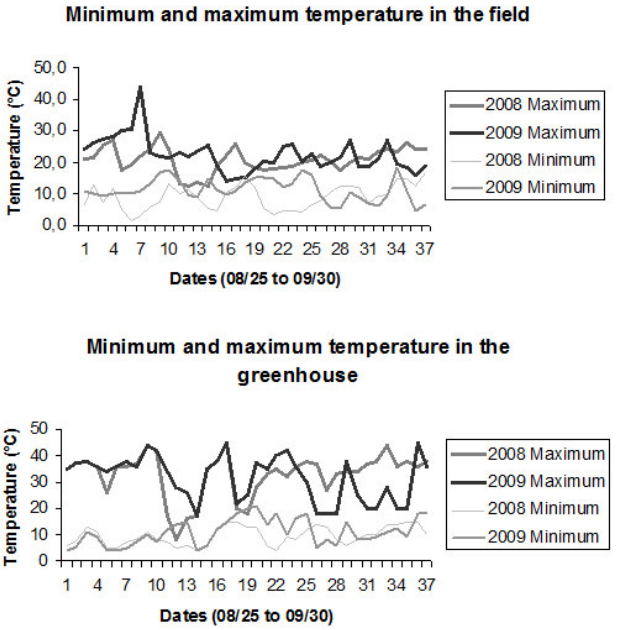

Figure 2. Minimum and maximum temperatures in the field and in the greenhouse from August 25 to September 30, 2008

Table 1. Meiosis in rootstocks under field and greenhouse conditions over three years

Rootstock	**Number of cells**	**Normal cells** (%)	**Number of cells**	**Normal cells** (%)	**Number of cells**	**Normal cells** (%)
	2008		2009		2010	
Field						
Trifoliate	188	62.77 aA	231	48.48 aA	225	61.78 aA
'Swingle'	180	57.22 cA	246	40.24 cA	226	57.52 bA
'Troyer'	173	61.85 abA	229	46.72abA	214	61.21 aA
'Fepagro C13'	204	60.29 abA	200	45.00 bA	228	60.96 abA
'Fepagro C37'	191	58.12 bcA	225	41.78 cA	235	59.15 abA
Averages	-----	60.05	-----	44.44	-----	60.12
Greenhouse						
Trifoliate	176	58.52 aB	170	35.29 aB	236	55.93 aB
'Swingle'	164	46.95 cB	235	28.94 cB	207	51.69 bB
'Troyer'	202	56.93 abB	208	31.73 bB	231	55.84 aB
'Fepagro C13'	178	56.74 abB	200	31.00 bB	226	54.42 aB
'Fepagro C37'	275	51.64 bB	230	30.43 bcB	258	52.71 bB
'Fepagro C41'	221	45.70 c	219	28.31 c	270	46.30 c
Averages	-----	52.75	-----	30.95	-----	52.82

Averages followed by the same lower case letter in the column do not differ significantly, for the same condition, by the Tukey test ($p>0.05$). Averages followed by the same capital letter in the column do not differ significantly in comparison with the same rootstock under different conditions by the Tukey test ($p>0.05$).

Table 2. Meiotic index (%) in rootstocks under field and greenhouse conditions over three years

Rootstock	**Number of cells**	**Meiotic Index** (%)	**Number of cells**	**Meiotic Index** (%)	**Number of cells**	**Meiotic Index** (%)
	2008		2009		2010	
Field						
Trifoliate	1004	64.24 aA	1001	52.35 aA	1022	64.09 aA
'Swingle'	1000	58.70 bA	1169	41.49 cA	1040	58.56 bA
'Troyer'	1066	62.10 abA	1025	47.90 bA	1018	62.38 abA
'Fepagro C13'	1165	61.72 abA	1055	46.54 bA	968	61.78 abA
'Fepagro C37'	1001	59.64 bA	1001	43.28 cA	1010	59.60 bA
Averages	-----	61.28	-----	46.31	-----	61.28
Greenhouse						
Trifoliate	1133	62.14 aA	1122	35.29 aB	1156	62.63 aA
'Swingle'	1022	47.36 dB	1169	30.80 bB	1000	47.70 cB
'Troyer'	1109	60.60 abA	1201	31.31 bB	1038	60.12 aA
'Fepagro C13'	1011	54.50 cB	1031	33.17 abB	1055	54.79 bB
'Fepagro C37'	1000	54.20 cB	1205	33.03 abB	1067	54.73 bB
'Fepagro C41'	1046	46.75 d	1066	26.92 c	1156	46.83 c
Averages	-----	54.26	-----	31.75	-----	54.47

Averages followed by the same lower case letter in the column do not differ significantly, for the same condition, by the Tukey test ($p>0.05$). Averages followed by the same capital letter in the column do not differ significantly in comparison with the same rootstock under different conditions by the Tukey test ($p>0.05$).

Table 3. Pollen viability (%) in rootstocks under field and greenhouse conditions over three years, and pollen *in vitro* germination and pollen tube length in rootstocks under field and greenhouse conditions in 2010

Rootstock	**Pollen fertility (%)**						**Pollen in vitro germination**	
	No. cells	**Viability** (%)	**No. cells**	**Viability** (%)	**No. cells**	**Viability** (%)	**Germin.** (%)	**Tube Length** (μm)
	2008		2009		2010		2010	
Field								
Trifoliate	10008	95.12 aA	10006	63.47 aA	10101	97.76 aA	47.49 aA	48.58 aA
'Swingle'	10064	85.46 dA	10165	49.32 dA	10157	55.11 dA	35.92 cA	44.30 abA
'Troyer'	10126	93.11 abA	10068	59.55 bA	10199	79.52 bA	46.52 aA	46.70 abA
'Fepagro C13'	10122	90.47 bcA	10050	57.12 bcA	10115	77.22 bA	44.00 bA	45.98 abA
'Fepagro C37'	10119	87.24 cdA	10330	51.72 dA	10203	64.08 cA	35.12 cA	43.30 bA
Averages	-----	90.28	-----	56.23	-----	74.74	41,81	45.59
Greenhouse								
Trifoliate	10176	68.13 bcB	10130	49.45 aB	10072	78.40 aB	43.12 aB	24.36 aB
'Swingle'	10303	52.08 dB	10335	36.70 cB	10097	57.96 dA	31.44deB	19.43 bcB
'Troyer'	10514	71.15 bB	10051	48.62 aB	10080	71.39 bB	41.56 bB	23.60 abB
'Fepagro C13'	10118	75.46 aB	10290	42.69 bB	10197	68.54 bcB	38.23 cB	23.17 abB
'Fepagro C37'	10024	65.31 cB	10226	38.41 cB	10159	61.11 cA	32.40 dB	21.89 abcB
'Fepagro C41'	10231	53.42 d	10126	32.60 d	10196	62.83 c	31.18 e	19.24 c
Averages	-----	64.25	-----	41.41	-----	66.71	36.32	21.78

Averages followed by the same lower case letter in the column do not differ significantly, for the same condition, by the Tukey test ($p>0.05$). Averages followed by the same capital letter in the column do not differ significantly in comparison with the same rootstock under different conditions by the Tukey test ($p>0.05$).

affecting these characteristics. Abiotic stresses such as water deficiency, nutrition, light, salinity, pollution and temperature are known to impair meiosis and pollen viability (Lalonde et al. 1997, Saini 1997, Sun et al. 2004). In the present study, we suggest that the greater amplitude of temperature variation in the greenhouse compared with the field is the main factor responsible for the observed abnormalities (Figure 2, data for 2008 and 2009).

For pollen *in vitro* germination, the experimental temperature used (25 ± 2 °C) is the one described as ideal for pollen germination in citrus (Cavalcante et al. 2000, Distefano et al. 2012). Differences in pollen germination between plants grown in the greenhouse (lower values) and those grown in the field (higher values) were found (Table 3). Even though temperature in the greenhouse was not monitored in 2010, oscillations like those in 2008 and 2009 were expected, which could explain the lower values of pollen viability and therefore lower pollen germination. According to Srinivasan et al. (1999), temperature stress during pollen grain formation may impair accumulation of energy and nutrients such as carbohydrates and amino acids, reducing germination ability. In pepper (*Capsicum annuum* L.), Aloni et al. (2001) found less pollen viability and germination under higher temperatures. Hedhly et al. (2004) in cherry (*Prunus avium* L.) and Nava et al. (2009) in peach (*Prunus persica* L.) observed lower pollen viability and germination in plants grown in protected environments compared with those grown in the field and associated their results with physiological damage caused by higher temperatures in the greenhouse.

Values for the characteristics analyzed were lower for both greenhouse and field in 2009 than in the other two years (Tables 1, 2 and 3). It may be concluded that in 2009 environmental conditions were more unfavourable in the greenhouse and in the field. Moreira and Gurgel (1941) already reported that different climatic conditions among years affect meiosis and pollen grain viability. Temperature variation and precipitation in the field during the flowering months (August and September), which was much higher in 2009 (618.5 mm) than in 2008 (308.4 mm) and 2010 (304.9mm), may have affected the results. Therefore, the factor responsible for that remains to be identified.

CONCLUSIONS

There was an unfavourable effect of greenhouse conditions on meiosis, meiotic index, pollen viability and *in vitro* germination compared with field conditions. It is possible that the greater temperature variation in the greenhouse was the causative factor.

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Reproductive characteristics of citrus rootstocks grown under greenhouse and field environments

Resumo – *O objetivo do presente trabalho foi avaliar o possível efeito dos fatores ambientais no processo meiótico, índice meiótico, viabilidade de pólen e germinação in vitro do pólen de plantas-matrizes dos porta-enxertos Trifoliata, 'Swingle', 'Troyer', 'Fepagro C13', 'Fepagro C37' e 'Fepagro C41' conduzidos em ambiente protegido em comparação com plantas-matrizes conduzidas a campo. Os resultados mostraram que os valores para as características analisadas em 2008, 2009 e 2010, foram sempre maiores no campo do que em casa-de-vegetação. A campo, a média de células meióticas normais foi de 60,05%, 44,44% e 60,12%, respectivamente e em casa-de-vegetação foi de 52,75%, 30,95% e 52,82%, respectivamente; a viabilidade média do pólen a campo foi de 90,28%, 56,23% e 74,74% e em casa-de-vegetação foi de 64,25%, 41,41% e 66,71%, respectivamente. Como as oscilações de temperatura foram maiores na estufa que, no campo, é sugerido que esta afeta negativamente as características reprodutivas analisadas.*

Palavras-chave: *Citricultura, comportamento meiótico, fertilidade do pólen, germinação do pólen.*

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