

## Effect of mechanical treatments on *in vitro* germination of citrus seeds

Rita de Cássia Mendes<sup>1</sup>  
Márcio Antônio Rocha de Oliveira<sup>1</sup>  
Sérgio Yoshimitsu Motoike<sup>1</sup>  
Rodrigo Sobreira Alexandre<sup>1</sup>  
Dalmo Lopes Siqueira<sup>1</sup>

### ABSTRACT

The present study aimed to show the effect of mechanical treatments applied to seeds of four citrus species, *Citrus sunki* Hort. Ex Tan., *C. limonia* Osb., *C. reshni* Hort. Ex Tan. and *Poncirus trifoliata*, and the hybrid *C. paradisi* Macf. x *P. trifoliata*, aiming at improving *in vitro* germination. Mechanical treatments consisted of seed coat removal and splitting of the endosperm into two halves. The evaluated parameters included percentage of germination, germination speed index, mean height of plantlets, and number of plantlets per seed. The results showed that mechanical treatments applied to seeds are beneficial to *in vitro* germination of *C. paradisi* x *P. trifoliata*, *C. reshni* and *P. trifoliata*. The treatments improved the percentage of seed germination as well as germination speed index and mean height of plantlets in these species. The endosperm division proved to be the best treatment applied to seeds and therefore it is recommended for improving *in vitro* germination of these species. However, *C. sunki* and *C. limonia* presented good *in vitro* germination performance without any treatment, showing that these species do not require the physical treatment for germination.

**Key words:** Tissue culture, propagation, vigor, testa, speed of germination.

### RESUMO

#### Germinação *in vitro* de sementes de citrus afetada por tratamentos mecânicos

O presente estudo trabalho objetivou mostrar o efeito de tratamentos mecânicos aplicado a sementes de quatro espécies cítricas, *Citrus sunki* Hort. Ex Tan., *C. limonia* Osb., *C. reshni* Hort. Ex Tan. e *Poncirus trifoliata*, e do híbrido *C. paradisi* Macf. x *P. trifoliata*, para melhorar a germinação *in vitro*. Os tratamentos mecânicos consistiram em remoção do tegumento da semente e divisão do endosperma ao meio. Os parâmetros avaliados foram a porcentagem de germinação, o índice de velocidade de germinação, o comprimento de plântulas e o número de plântulas por semente. Foi concluído que tratamentos mecânicos aplicados às sementes são benéficos para germinação *in vitro* de *C. paradisi* x *P. trifoliata*, *C. reshni* e *P. trifoliata*. Os tratamentos melhoraram a porcentagem de germinação de semente, bem como o índice de velocidade de germinação e o comprimento de plântulas destas espécies. A divisão do endosperma provou ser o melhor tratamento aplicado às sementes e, portanto, o indicado por melhorar a germinação *in vitro* destas espécies. Porém, *C. sunki* e *C. limonia* apresentaram bom desempenho de germinação *in vitro* sem qualquer tratamento, mostrando que estas espécies não requerem tratamento para germinação.

**Palavras-chave:** cultura de tecidos, propagação, vigor, testa, velocidade de germinação.

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<sup>1</sup> Universidade Federal de Viçosa, departamento de Fitotecnia, Av. P. H. Rolfs, s/n. 36570-000 - Viçosa, MG. E-mails: mendescrita@yahoo.com.br, moliveira@ufv.br, motoike@ufv.br, rsalexandre@click21.com.br, siqueira@ufv.br. Apoio financeiro CNPq, CAPES

## INTRODUCTION

Brazil is the largest producer of citrus fruits in the world, contributing with more than 23 million tons per year (FAO, 1998). For the last 30 years, increase in citrus production has been related to the expansion of the cultivated lands, which have been growing throughout the national territory, and therefore having great economic and social impact.

Citrus species have been vegetatively propagated by rootstock. Citrus are therefore susceptible to numerous diseases transmitted by contaminated scions. Viral diseases such as tristeza-virus, exocortis, sorosis and xyloporosis debilitate the plant leading to decrease in both production and useful life of orchards. Considering that the control of these diseases is based on prevention, it is crucial to use healthy seedlings, from virus-free propagative material.

Clonal viral-elimination may be accomplished through the *in vitro* micrografting technique, firstly applied to citrus by Navarro *et al.* (1975). This technique consists of *in vitro* germination of rootstock seeds; then vitroplants are later obtained, decapitated and grafted through a small meristematic growing tip consisting of the meristem plus two leaf primordia. However, the *in vitro* germination rate of rootstocks, especially 'Citrumelo' (*Citrus paradisi* Macf. x *Poncirus. trifoliata*) and 'Trifoliata' (*P. trifoliata*) are quite low, reaching below 50%. There is, therefore, a need for improving the *in vitro* germination of these seeds. According to Soares Filho *et al.* (2002), the removal of both internal and external seed teguments can enhance citrus germination. This is nevertheless a very time consuming procedure, considering that it should be performed under aseptic conditions in order to avoid contamination of the culture.

This study aimed to test different physical seed treatments, in order to improve *in vitro* germination of citrus species and hybrids used as rootstock.

## MATERIALS AND METHODS

The present study was carried out at the Plant Tissue and Cell Culture Laboratory of the Plant Science Department, Universidade Federal de Viçosa (UFV), Viçosa -MG. Seeds extracted from fruits were treated with quicklime for the removal of mucilage, washed in tap water on a fine-mesh sieve. After washing, seeds were dried on paper towel for 24 hours in the shade, except for the 'Citrumelo' seeds that were used immediately after extraction, because their recalcitrant characteristics (Siqueira *et al.*, 2002).

The experiment consisted of 20 treatments arranged in a complete randomized design in a 5 x 2 x 2 factorial scheme, with four replicates of 25 seeds each. The following factors were studied: five citrus genotypes, two physical

treatments applied to seed teguments, and two physical treatments applied to seed endosperms.

The studied species were tangerine 'Sunki' (*Citrus sunki* Hort. ex Tan.), rangpur lime Cravo (*Citrus limonia* Osb.), tangerine 'Cleópatra' (*Citrus reshni* Hort. ex Tan.) and 'Trifoliata' (*P. trifoliata*), and the hybrid 'Citrumelo' (*Citrus paradisi* Macf. x *Poncirus. trifoliata*). The physical treatment applied to the tegument consisted of total manual removal or not performed before seed disinfection, whereas the physical treatments applied to the endosperm were performed in a laminar flow cabinet after seed disinfection, consisting of splitting endosperm or not, with a scalpel. Seeds were surface sterilized with ethanol 70% (v/v) for one minute, followed by immersion in sodium hypochlorite solution with 2.0-2.5% active chlorine, for 20 minutes. After each treatment, seeds were rinsed four times in sterile deionized distilled water. Seeds were then germinated in medium consisted of MS salts (Murashige and Skoog, 1962), White (1951) vitamins, 8 g L<sup>-1</sup> agar Isofar® as solidifying agent, pH adjusted to 5.7 ± 0.1 before adding the agar melted in microwave oven. Ten-ml aliquotes of medium were poured into 25 x 150-mm test tubes that were covered with transparent polypropylene caps and autoclaved at 120 °C, 1.5 kg cm<sup>-2</sup> for 20 minutes. Seeds were placed into test tubes (one seed per tube) and incubated in growth room, at 27 ± 2 °C, photoperiod of 16/8 h (light/dark) and irradiance of 60 μmol m<sup>-2</sup> s<sup>-1</sup> for 40 days.

Seed germination was monitored daily and germination speed was obtained (Maguire, 1962). After 40 days, percentage of seed germination was determined, plantlets were removed from the test tubes for evaluation of mean height and number of plantlets per seed. Data were examined by analysis of variance and means compared by the Tukey test at 5% probability.

## RESULTS AND DISCUSSION

In the present study, *in vitro* seed germination was obtained for the citrus cultivars 'Citrumelo' (*Citrus paradisi* Macf. x *Poncirus. trifoliata*), tangerine 'Sunki' (*C. sunki* Hort. ex Tan.), rangpur lime 'Cravo' (*C. limonia* Osb.), tangerine 'Cleópatra' (*C. reshni* Hort. ex. Tan.) and 'Trifoliata' (*P. trifoliata*). All these cultivars germinated in MS medium lacking growth regulators, independently of the applied treatment. However, the germination percentage, GSI (*germination speed index*) and the mean plantlet height varied significantly as a function of the cultivar and the treatment applied on both tegument and endosperm (Figures 1, 2 and 3).

### Seed germination percentage

Among the studied cultivars, rangpur lime 'Cravo' and tangerine 'Sunki' presented higher percentages for *in vitro*

germination (98%), without the need for physical treatments. However, tangerines 'Cleópatra', 'Citrumelo' and 'Trifoliata' showed low germination percentages (21, 9 and 4%, respectively), showing therefore the need for seed treatment (Figure 1).

Among the physical treatments, splitting the endosperm into two halves provided the best results. Seed germination percentages of tangerine 'Cleópatra', 'Citrumelo' and 'Trifoliata' showed increase of 79, 83 and 84%, respectively (Figure 1). Removing seed tegument also increased the germination of these cultivars, but at a lower rate. The increments in the germination percentages were 54.35 and 10%, respectively. No positive interaction was observed between the effect of physical treatment on tegument and the effect of physical treatment on endosperm; thus, in this case, there are no advantages in combining both treatments.

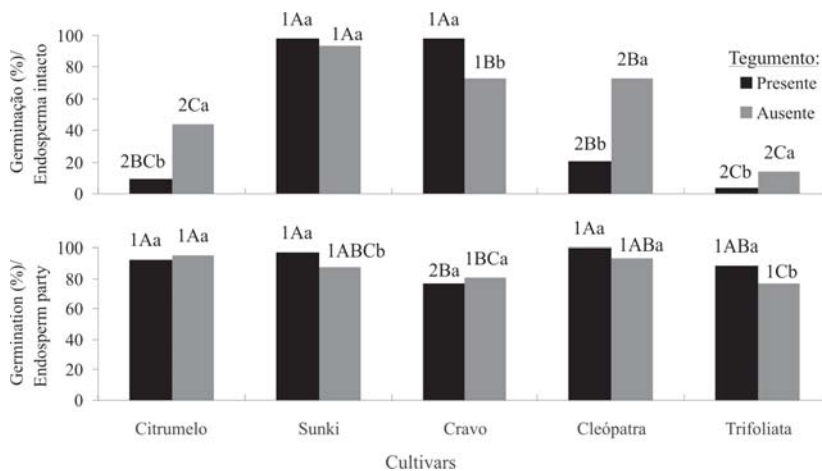
High germination of tangerine 'Sunki' was recorded in this work, even with no seed treatment. Soares Filho *et al.* (1995) discussed that natural ruptures in the external and internal seed teguments make it possible a higher oxygen diffusion and water uptake by the seed, increasing germination. Therefore, the increment obtained in

germination rates by halving the seed would be probably due to the rupture of the natural mechanical barriers that block the uptake of water and oxygen diffusion (Popinigis, 1985). These barriers consist of structures such as wax cuticle, suberine, palisade tissues, and macrosclereid layers found in seeds (Gualtieri and Peres, 2004)

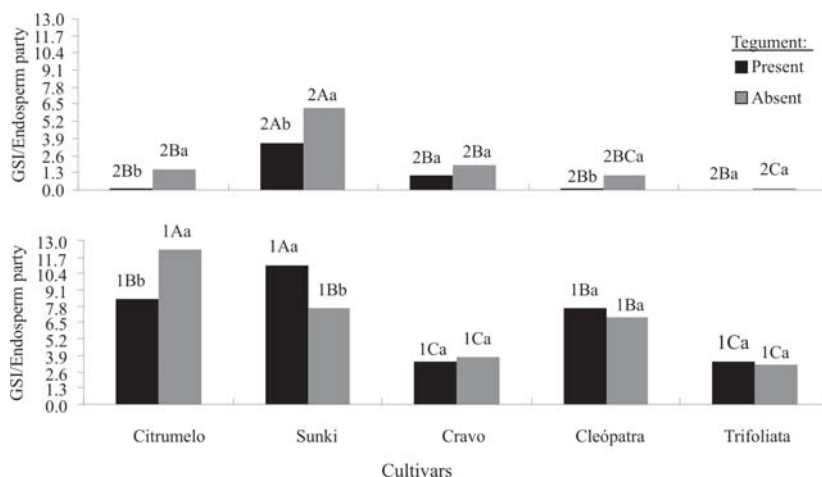
### Germination speed index (GSI)

Germination speed of citrus rootstock seeds varied significantly as a function of species and treatment applied to tegument and endosperm. Among the cultivars tested with no treatments, tangerine 'Sunki' showed the highest *in vitro* GSI (3.53 points), followed by rangpur lime 'Cravo' (1.16 points). GSI for the other cultivars was below 0.2 points (Figure 2).

Splitting of the seeds into two halves provided the highest acceleration of the *in vitro* germination process in citrus. This treatment increased by 8.35, 7.53, 7.48, 3.34 and 2.20 points GSI of 'Citrumelo', tangerine 'Cleópatra', tangerine 'Sunki', 'Trifoliata' and rangpur lime 'Cravo' seeds, respectively, when compared with untreated seeds (Figure 2). The effect of tegument removal was only significant for tangerine 'Sunki', 'Citrumelo' and tangerine



**Figure 1.** Effect of physical treatments of seed tegument and endosperm on *in vitro* germination (%) of citrus seeds. \* Means followed by the same letters, capital letters for cultivars and small letters for bars, in the same graph, and numbers between graphs, are not significantly different at 5% probability by the Tukey test.



**Figure 2.** Effect of physical treatments of seed tegument and endosperm on *in vitro* germination speed of citrus seeds. \* Means followed by the same letters, capital letter for cultivars and small for bars, in the same graph, and numbers between graphs, are not significantly different at 5% probability by the Tukey test.

'Cleópatra', with GSI increasing 2.67, 1.41 and 0.93 points, respectively. A positive interaction occurred between the effects of endosperm splitting and tegument removal for the cultivar 'Citrumelo'. The combined application of these treatments increased by 12.10 points the GSI of this cultivar compared with untreated seeds; therefore, this combined application is recommended for *in vitro* germination of this species.

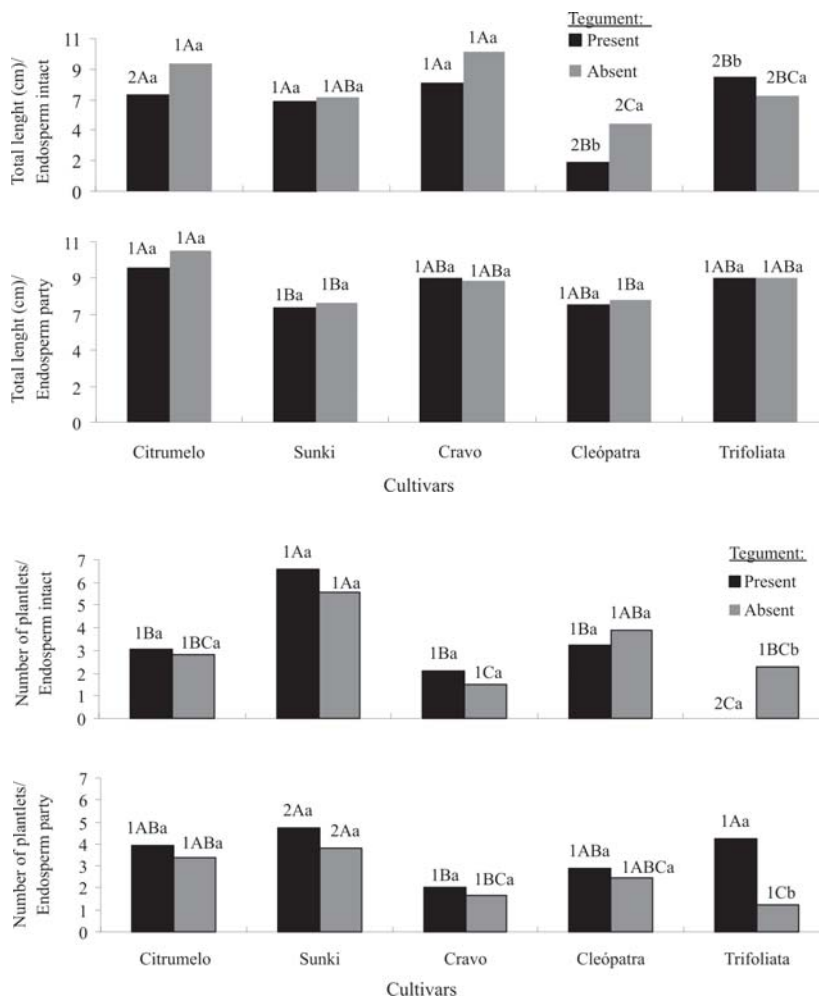
The acceleration of germination induced by the physical treatment is probably due to a greater contact of the embryo with the culture medium and oxygen. In this case, the physical treatment would have broken the natural barriers in the seed, exposing the embryo more rapidly to a higher amount of water and nutrients, resulting in an accelerated germination. Because the *in vitro* environment is sterile, this exposure occurred without the risk of infections to the embryo. Soares Filho *et al.* (2002) observed that the removal of the external tegument (testa) or both external and internal teguments (tegmen) of citrus seeds anticipated considerably the beginning of germination and increased the emergence speed of plantlets.

### Total plantlet height

The total height of plantlets varied with the species or physical treatments applied to seed tegument and endosperm (Figure 3). Among all treatments, again the physical treatment applied to endosperm showed better results, since the heights of 'Citrumelo', tangerine 'Cleópatra' and 'Trifoliata' plantlets were 2.5, 5.1 and 0.6 cm, respectively, thus higher than the plantlets originated from the untreated seeds. This advantage might be caused by a higher germination speed of treated seeds (Figure 2), providing the plantlets originated from these seeds with a longer time for their development.

### Average plantlet number per se

Plantlet number varied mainly as a function of the genotype, and tangerine 'Sunki' showed the highest number of plantlets per seed (Figure 4). This was an expected result, since the number of plantlets is directly related to the polyembryonic degree of seeds. Tangerine 'Sunki' seeds are widely known for its high polyembryonic nature (Parlevliet and Cameron, 1959; Cameron and Soost, 1979).



**Figure 3.** Effect of physical treatments of seed tegument and endosperm on total height (cm) of *in vitro* citrus plantlets. \*Means followed by the same letters, capital letter for cultivars and small for bars, in the same graph, and numbers between graphs, are not significantly different at 5% probability by the Tukey test.

**Figure 4.** Effect of physical treatments of seed tegument and endosperm on number of *in vitro* plantlets developed from citrus seeds. \* Means followed by the same letters, capital letter for cultivars and small for bars, in the same graph and numbers between graphs, are not significantly different at 5% probability by the Tukey test.

The treatments applied to seeds affected significantly tangerine 'Sunki' and 'Trifoliata' (Figure 4). The number of tangerine 'Sunki' plantlets decreased with the application of physical treatment to endosperm, probably because the damages caused by halving the endosperm. 'Trifoliata' plantlets increased with the splitting of endosperm and removal of tegument. These treatments, however, should be applied separately, as they caused a reduction in the number of plantlets per seed in this species when they were combined. In this case, the best result was obtained by splitting the endosperm, which increased by 4.25 the mean of plantlets per seed of 'Trifoliata'.

## CONCLUSIONS

The results of the present study led to the following conclusions: physical treatments applied to 'Citrumelo', tangerine 'Cleópatra' and 'Trifoliata' seeds were beneficial to *in vitro* germination, as they increased germination percentage, germination speed and total height of plantlets. Among the treatments, splitting of endosperm gave the best results, and it is recommended to improve *in vitro* seed germination of these species. The cultivars rangpur lime 'Cravo' and tangerine 'Sunki' showed good performance for *in vitro* seed germination, therefore, with no need for treatments of seeds of these species.

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