

# Embryo culture and *in vitro* clonal multiplication of *Prunus* 'Capdeboscq' rootstock

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## ABSTRACT

Embryo culture and recovery are techniques used in basic and applied studies of morphogenesis, *in vitro* propagation and genetic breeding in the *Prunus* genus. The present study assessed the germination and organogenetic development of embryos in culture media with different concentrations of GA<sub>3</sub> as well as the *in vitro* multiplication rate of clones of the 'Capdeboscq' rootstock. Embryonic axes were excised and inoculated in Lepoivre culture medium supplemented with BAP (0.1 mg L<sup>-1</sup>), NAA (0.1 mg L<sup>-1</sup>) and GA<sub>3</sub> (0.0; 1.0; 2.5; 5.0; 7.5 and 10.0 mg L<sup>-1</sup>). The *in vitro* multiplication potential of four clones was assessed after embryo germination using the double phase multiplication method in the last subculture. The results showed a mean of 70.7% *in vitro* germination. GA<sub>3</sub> was not effective in the germination, but induced greater elongation of the embryo and inhibited the root development. The clones obtained in the embryo culture showed high potential for *in vitro* multiplication with rates of 6.9 and 14.9 shoots per explant.

**KEY WORDS:** Peach tree, gibberellic acid, germination, organogenesis, *in vitro* propagation.

## INTRODUCTION

Peach tree cultivation is of great social and economic importance in the South and Southeast regions of Brazil. Rio Grande do Sul is the main producing state, with a cultivated area of 11.9 thousand hectares and fruit production of 53 thousand tons. Santa Catarina is the second state in area and the third in production with 4.5 thousand hectares and 38.9 tons of fruit, respectively (Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina, 2001).

Most of the commercial peach orchards in the Southern region were implanted with cuttings grafted on rootstocks obtained from seeds from the preserves industry. Under these conditions there are mixtures of varieties, sanitary problems, early death and especially, lack of genetic identity (Fachinello, 2000).

The rootstocks used in peach and plum tree cutting production are derived from the Capdeboscq cultivar (*Prunus persica* (L) Batsch). This cultivar originated in the Experimental Station of Pelotas, RS, from open pollination of a cross between 'Lake City' and a local selection called 'Intermediario' (Finardi, 1998). This cultivar is late cycle and produces fruit for industry. Its seeds have high fertilization rates, a characteristic that is not very common in the other peach tree cultivars.

New propagation technologies based on culture techniques of plant tissue are required for modern

fruit production, especially the rootstocks, that allow clonal and mass propagation from elite plants with genetic fidelity and sanitary control (Damiano and Palombi, 2000).

Embryo culture technique has been used in peach trees for studies on *in vitro* regeneration and genetic transformation (Pooler and Scorza, 1995; Scorza et al., 1995). It has also been used to recover immature embryos in hybridizations among peach tree parents with early maturity, yielding embryos with incomplete development at the fruit maturation phase (Feliciano and Assis, 1989; Navarro et al., 1992; Quezada et al., 1998).

Zygotic embryos are an excellent source of explants for *in vitro* culture because of their juvenile trait and high regenerative potential (Burgos and Ledbetter, 1993; Pooler and Scorza, 1995). Thus the embryo culture technique stands out as an excellent alternative to develop germination protocols, *in vitro* establishment and propagation and also allows the optimization of culture media for different genotypes.

Among the factors that affect the *in vitro* morphogenetic responses in the embryo culture and recovery technique, gibberellic acid (GA<sub>3</sub>) is known for its effects on the stimulus to germination of dormant embryo *in vitro* growth and development and is mainly associated with cell division and elongation (Rouskas et al., 1980; Dalzotto and Docampo, 1997;

Hu and Ferreira, 1998).

The main objective of the present study was to evaluate the effect of GA<sub>3</sub> on *in vitro* germination and on the organogenetic development of embryos of the *Prunus* 'Capdeboscq' rootstock and to assess the *in vitro* multiplication rate of clones obtained from embryo culture.

## MATERIAL AND METHODS

'Capdeboscq' seeds were collected at Epagri – Videira Experimental Station (Videira – SC). The seeds without the endocarps were kept for six months in a cold chamber at 4 °C and later disinfected with 70% ethanol (2 min), 750 mg L<sup>-1</sup> tetracycline (Tetrex®) (24 h), 1.0 g L<sup>-1</sup> benomyl (Benlat®) (10 min) and 1.25% sodium hypochloride (40 min).

The embryonic axes were washed three times in sterilized distilled water, placed in a flow chamber, excised and inoculated in test tubes (25 x 150mm) containing 10 mL Lepoivre culture medium (Quoirin et al., 1977), supplemented with sucrose (20.0 g L<sup>-1</sup>), ágar (7.0 g L<sup>-1</sup>), BAP - 6 benzylaminopurine (0.1 mg L<sup>-1</sup>), NAA - a naphthaleneacetic acid (0.1 mg L<sup>-1</sup>) and GA<sub>3</sub> – gibberellic acid (0.0; 1.0; 2.5; 5.0; 7.5 and 10.0 mg L<sup>-1</sup>). The culture medium pH was adjusted to 5.2-5.3 before autoclaving at 121 °C for 15 minutes.

The cultures were evaluated 30 days later for contamination percentage, germination percentage, stem height, percentage of root system development and number of roots per embryo. Germinated embryos were considered to be those that developed the stem apical meristem.

After germination the stem apical meristem of the

embryo was removed and underwent the multiplication phase in the same culture medium mentioned previously but supplemented only with BAP (0.5 mg L<sup>-1</sup>). After two subcultures every 21 days, four clones were randomly selected for *in vitro* multiplication, called CB1, CB3, CB4, and CB5. At the third subculture, 1-2 cm nodal segments of these clones were submitted to *in vitro* culture in double phase culture medium with the solid phase consisting of Lepoivre culture medium (Quoirin et al., 1977) supplemented with BAP (0.5 mg L<sup>-1</sup>). After 15 days culture, the same culture medium liquid but without BAP was added to the solid phase.

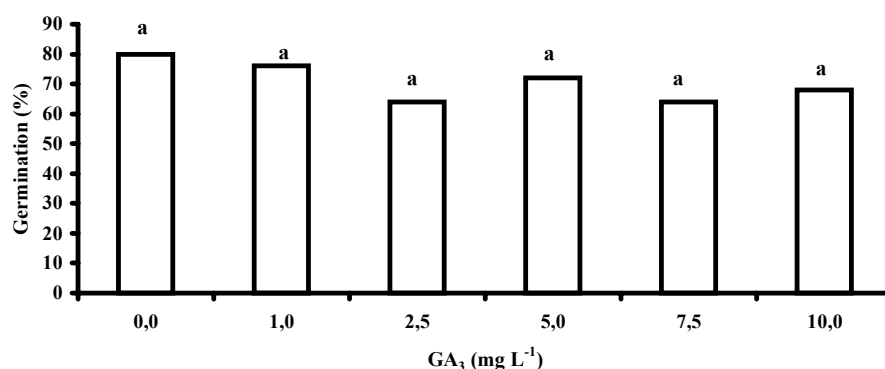
The cultures were assessed after 30 days for the number of shoots per explant, mean shoot height (mm) and number of shoots greater than 20 mm.

The different culture phases were kept in a growth chamber at 25 ± 2 °C, 16 hour photoperiod and light intensity of 40-45 mmol.m<sup>-2</sup>.s<sup>-1</sup>, supplied by cold white fluorescent lamps.

A complete randomized block experimental design was used for the embryos with five embryonic axes per replication and five replications per treatment. Three replications per treatment and five plants per replication were used at the clone multiplication phase. All the data were submitted to analysis of variance (ANOVA) and to the SNK mean separation test (Sokal and Rohlf, 1995).

## RESULTS AND DISCUSSION

The results obtained showed that the *in vitro* recovery and cultivation process of the 'Capdeboscq' peach tree embryos were efficient. A mean rate of 14% of



**Figure 1.** *In vitro* germination percentage of *Prunus* 'Capdeboscq' rootstock embryo after 30 days in Lepoivre culture medium supplemented with BAP (0.1 mg L<sup>-1</sup>), NAA (0.1 mg L<sup>-1</sup>) and different levels of GA<sub>3</sub>. UFSC, Florianópolis-SC, 2002.

contaminated embryo was detected in the *in vitro* establishment (data not shown) values that were considered compatible with those observed by other authors, including Navarro et al. (1992) who observed variation from 0 to 14% in the contamination rate in the *in vitro* culture of peach tree embryos. For these authors, the removal of the seed coat presented positive effects in terms of reducing culture contamination.

The mean values obtained for the germination percentage were 70.7% (Figure 1), situated in the same range observed by Burgos and Ledbetter (1993) for apricot embryo culture and by Quezada et al. (1998) for nine peach tree cultivars.

No significant differences were observed among different GA<sub>3</sub> concentrations for the germination rate (Figure 1). However, a decrease in the germination rates was observed with the increase in the GA<sub>3</sub> concentrations supplemented in the culture media. Cicero (1986) stated that germination depends on physiological factors and on the levels and balances of the hormones present in the embryo. The ineffective results of GA<sub>3</sub> in the germination may be explained by the use of only the embryonic axis, thus assuming that the factors responsible for the dormancy of the embryo would be present in the seed coat and not in the embryo. According to Toit et al. (1979) the seed coat has an inhibiting effect on germination in the peach tree. Navarro et al. (1992) also pointed out that the maintenance of the seed coat in peach tree embryo culture determined a low germination rate and a reduction in the aerial part length.

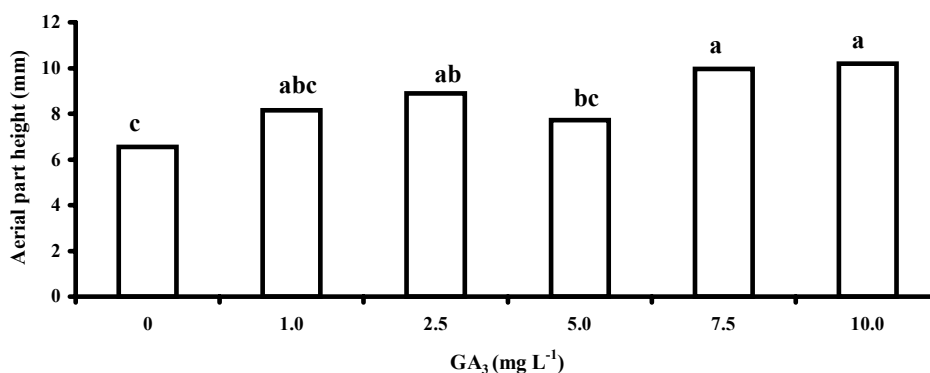
The results obtained in the present study showed a

positive effect of GA<sub>3</sub> on the growth of the stem apical meristem that increased proportionally to the GA<sub>3</sub> concentration used (Figures 2 and 5).

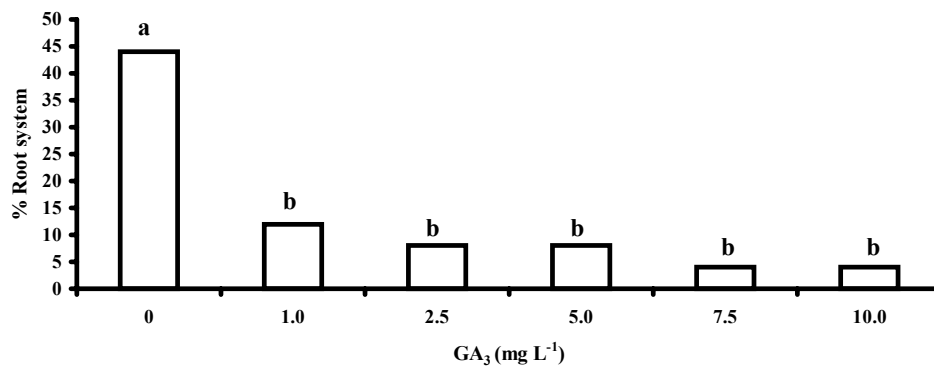
High GA<sub>3</sub> concentrations (*i.e.* 7.5 and 10.0 mg L<sup>-1</sup>) resulted in a 10 mm aerial length, significantly different to results observed in the GA<sub>3</sub> - free control (Figure 2). The elongation of the stem apical meristem is one of the more important effects of GA<sub>3</sub> in the *in vitro* culture process. The gibberellins are associated with cell elongation and division (Taiz and Zeiger, 1998). The embryo stem apical meristem height is an important factor for *in vitro* establishment and propagation because it makes the replication process easier in the multiplication. Reeves et al. (1985) reported positive results from GA<sub>3</sub> in the elongating *Prunus* 'St. Julien A' rootstock shoots. These authors reported that GA<sub>3</sub> concentrations lower than 12.5 mg L<sup>-1</sup> were not efficient for *in vitro* growth.

Significant differences were observed for the embryo percentage with root system and number of roots per embryo for the different GA<sub>3</sub> concentrations used (Figures 3 and 4). GA<sub>3</sub> impaired the root system development and reduced the number of roots per embryo. GA<sub>3</sub> concentrations up to 1.0 mg L<sup>-1</sup> significantly reduced the root system development from 44% to *c.* 4% (Figure 3). The addition of GA<sub>3</sub> to the medium resulted in a strong reduction in embryo root number, ranging from 2,8 in the control to 0.2 roots in response to 7.5 mg L<sup>-1</sup> (Figure 4).

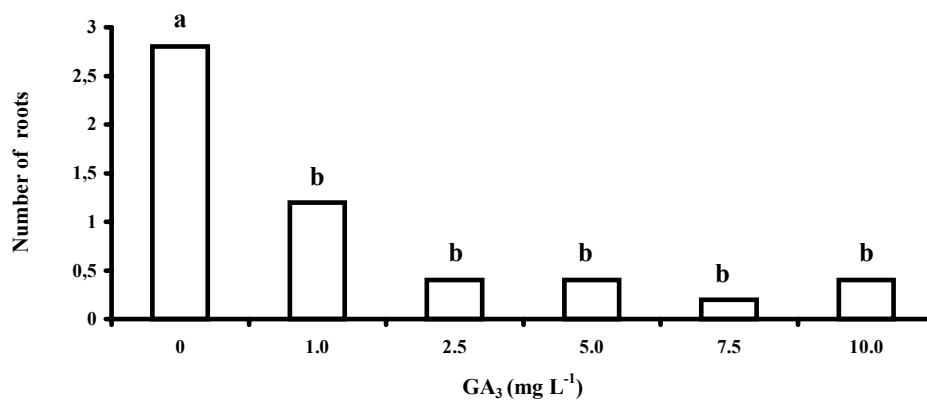
Several authors have reported results of root development inhibition in the presence of GA<sub>3</sub>. According to Feliciano and Assis (1989) in spite of the negative effect of GA<sub>3</sub> on embryo root development, aerial part growth and absence of roots



**Figure 2.** Mean aerial part height (mm) in *in vitro* embryos *Prunus* 'Capdeboscq' rootstock embryo after 30 days in Lepoivre culture medium supplemented with BAP (0.1 mg L<sup>-1</sup>), NAA (0.1 mg L<sup>-1</sup>) and different levels of GA<sub>3</sub>. UFSC, Florianópolis-SC, 2002.



**Figure 3.** Percentage of *in vitro* root system development of embryos *Prunus* 'Capdeboscq' rootstock embryo after 30 days in Lepoivre medium supplemented with BAP (0.1 mg L<sup>-1</sup>), NAA (0.1 mg L<sup>-1</sup>) and different levels of GA<sub>3</sub>. UFSC, Florianópolis-SC, 2002.



**Figure 4.** Number of *in vitro* roots *Prunus* 'Capdeboscq' rootstock embryos after 30 days in Lepoivre culture medium supplemented with BAP (0.1 mg L<sup>-1</sup>), NAA (0.1 mg L<sup>-1</sup>) and different levels of GA<sub>3</sub>. UFSC, Florianópolis-SC, 2002.

in peach tree embryo culture is not uncommon. Reeves et al. (1985) reported that the 12.5 mg L<sup>-1</sup> level of GA<sub>3</sub> used to stimulate the lengthening of the *Prunus insitia* the growth of aerial part inhibited the rooting process.

Table 1 shows the results observed for the *in vitro* multiplication phase of the CB1, CB 3, CB4 and CB5 clones. The values observed were 6.9 and 14.9 shoots per explant and the CB3 and CB4 clones showed the highest multiplication rate, 14.9 and 13.1 shoots per explant, respectively.

These results are greater than those obtained by Parfitt and Almehdi (1986) and Hammerschlag et al. (1987) for 56 and 8 varieties of peach tree cultivated *in vitro*, respectively. The greater multiplication rate observed in the present study may be mainly related to the genotype and the formulation of the culture medium. The saline formulation developed by Quoirin et al. (1977) for *in vitro* culture of woody fruit trees presents reduced concentration of total nitrogen, reduction of

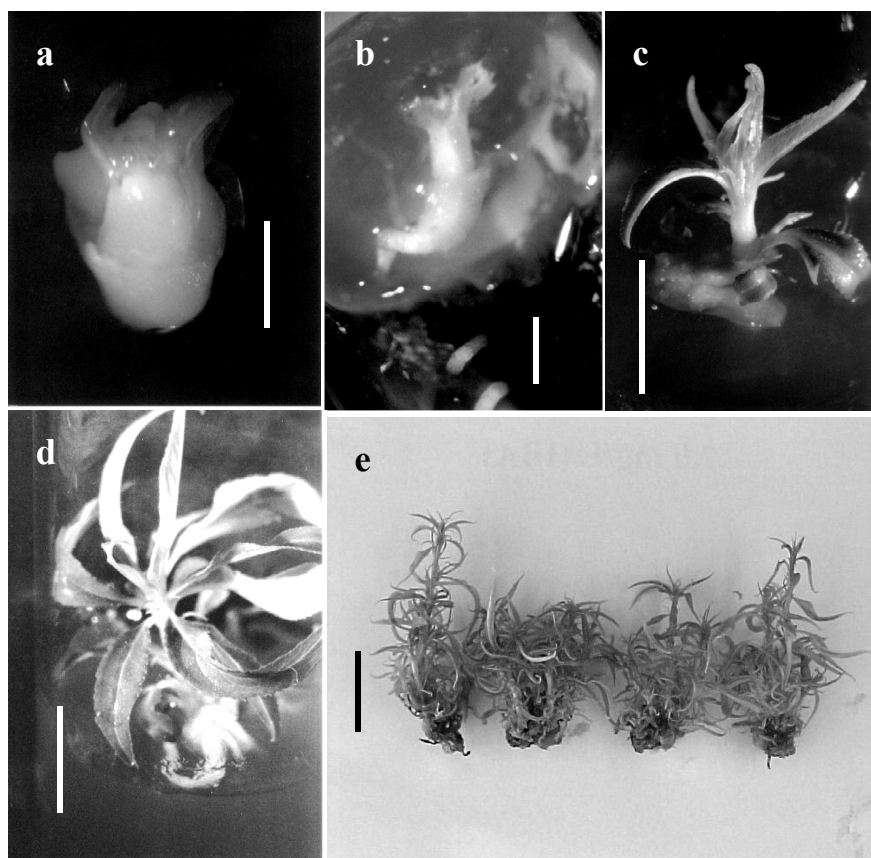
NH<sub>4</sub><sup>+</sup> ions, increase in Ca<sub>2</sub><sup>+</sup> ions and absence of Cl<sup>-</sup> ions as its main characteristics. These aspects have shown positive results for *in vitro* culture of different *Prunus* genus species, such as the increase in the multiplication rates and reduction of hyperhydric shoots (Bouza et al., 1992; Murai et al., 1997; Pérez-Tornero and Burgos, 2000).

There were also significant differences among the clones for the number of shoots greater than 20 mm, with values ranging 1.1 to 3.7 per explant. The CB3 clones, with 3.7 shoots per explant, were superior to the others, followed by the CB4 clone, with 2.8 shoots per explant. There were no significant differences among the clones for shoot height, and the mean values observed ranged from 10.9 to 14.3 mm (Figure 5). These results are similar to those observed by Parfitt and Almehdi (1986) who assessed 56 varieties of peach tree *in vitro*. They reported that mean shoot height values varied from 13.6 to 19.1 mm, in response to the AP culture medium supplemented

**Table 1.** Mean values of the number of shoots, mean shoot height (mm) and number of shoots greater than 20mm of *Prunus* ‘Capdeboscq’ rootstock clones *in vitro* multiplication after 30 days in Lepoivre double-phase culture medium. UFSC, Florianópolis-SC, 2002.

Clones	Number of shoots	Mean shoot height (mm)	Number of shoots > 20mm
CB1	9.9 b	12.6 a	1.6 c
CB3	14.9 a	14.3 a	3.7 a
CB4	13.1 a	13.2 a	2.8 b
CB5	6.9 c	10.9 a	1.1 c
CV(%)	11.0	12.5	16.5

Means followed by the same letters in the columns do not differ significantly by the SNK test at 5%.



**Figure 5.** Morphogenetic aspects of the *in vitro* development of ‘Capdeboscq’ rootstock embryos. A) embryonic axle established *in vitro* (bar 1mm). B) embryonic axis with bipolar development, 7 days *in vitro*, (bar 3mm). C) embryo with apical stem development, 15 days *in vitro* (bar 10mm). D) embryo with bipolar development, 30 days *in vitro* (bar 10mm). E) stem tips of embryo at *in vitro* multiplication phase (bar 10mm). UFSC, Florianópolis-SC, 2002.

with 6.0 mg L<sup>-1</sup> BAP and 0.01 mg L<sup>-1</sup> IBA.

The genotype had a significant effect for the *in vitro* multiplication rate, thus confirming that the genotype is one of the more influential factors in the *in vitro* propagation process of peach trees as reported by Parfitt and Almehdi (1986); Leontiev-Orlov et al. (2000) and Pérez-Tornero and Burgos (2000).

## CONCLUSIONS

The results obtained in the present study led to the conclusion that the technique of embryonic axes recovery and culture was efficient for *in vitro* germination, establishment and multiplication of the peach tree ‘Capdeboscq’. GA<sub>3</sub> at the levels used did

not showed a positive effect on the germination rate of the embryonic axes. However, this plant growth regulator activated the stem apical meristem growth and decreased *in vitro* root development. The 'Capdeboscq' clones, derived from embryo culture, presented high *in vitro* multiplication rates, indicating the potential of this technique for clonal micropropagation of this rootstock, its clones and selections.

## RESUMO

### Cultura de embrião e multiplicação *in vitro* de clones do porta-enxerto de *Prunus* 'Capdeboscq'.

No gênero *Prunus*, a cultura e o resgate de embriões são técnicas empregadas em estudos básicos e aplicados de morfogênese, propagação *in vitro* e melhoramento genético. No presente trabalho avaliou-se a germinação, o desenvolvimento organogenético de embriões em meios de cultura com diferentes concentrações de GA<sub>3</sub> e a taxa de multiplicação *in vitro* de clones do porta-enxerto de *Prunus* 'Capdeboscq'. Eixos embrionários foram excisados e inoculados em meio de cultura de Lepoivre, suplementado com BAP (0.1 mg L<sup>-1</sup>), NAA (0.1 mg L<sup>-1</sup>) e GA<sub>3</sub> (0.0; 1.0; 2.5; 5.0; 7.5 e 10.0 mg L<sup>-1</sup>). Após a germinação dos embriões avaliou-se o potencial de multiplicação *in vitro* de quatro clones, utilizando o método de multiplicação *dupla-fase*, na última subcultura. Os resultados mostraram percentagem média de germinação *in vitro* de 70.7%. O GA<sub>3</sub> não foi efetivo na germinação, no entanto, induziu maior alongamento da parte aérea dos embriões e inibiu o desenvolvimento do sistema radicular. Os clones obtidos da cultura de embriões demonstraram alto potencial para a multiplicação *in vitro*, com taxas de 6.9 a 14.9 brotos por explante.

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